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QUANTITATIVE ASSAY OF THE 3β-OL-DEHYDROGENASE SYSTEM IN WHOLE ADRENAL HOMOGENATE[1]

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SAMUELS and co-workers (1, 2) demonstrated the presence in endocrine tissues of a dehydrogenase which oxidizes Δ^5-3β-hydroxysteroids to the corresponding Δ^4-3-ketone. This enzyme acts upon both C_{19} and C_{21} steroids. It has been postulated (because of the increased urinary excretion of dehydroepiandrosterone) that the enzyme concentration is relatively low in adrenal cancer of the masculinizing type, although it is not believed to be changed in other types of adrenal disease (3, 4, 5). Whether the concentration of this enzyme may be influenced by other conditions known to change the steroid output of the adrenal, such as administration of ACTH, or imposition of stress, is also of significance.

To test these postulates experimentally, a quantitative assay method for 3β-ol-dehydrogenase in whole adrenal homogenate has been developed. The reaction utilized is the conversion of dehydroepiandrosterone to Δ^4-androstene-3,17-dione. The C_{19} steroid is used because the product is readily measured by the Zimmermann reaction. The method, and some applications, are described in this communication.

METHODS

Fresh or frozen adrenal glands were sliced and homogenized for one minute in a Potter-Elvehjem (6) or VirTis[3] homogenizer, in the ratio of 1 gm. of tissue to 1 ml. of a homogenizing medium consisting of equal parts of 0.9% sodium chloride and 0.1 M phosphate buffer (pH 7.4), then filtered through gauze. This homogenate contains approximately 1 gm. of tissue per 1.5 ml. Dilute homogenates, containing approximately 0.5, 0.25, 0.125, and 0.063 gm. of tissue per 1.5 ml. were made with homogenizing medium. The nitrogen content (mg. N) of each concentration was determined by a micro-Kjeldahl procedure (7).

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[3] Obtained from VirTis Company, Inc., 160 Ashburton Ave., Yonkers, N. Y.

1–1.5 mg. of dehydroepiandrosterone, dissolved in 0.06 ml. of propylene glycol, was incubated with 1.5 ml. of each homogenate concentration, in duplicate, in 20 ml. beakers. Tissue blanks were always incubated. Diphosphopyridine nucleotide (DPN) (3.4 mg. in 0.2 ml. buffer) was added to each to ensure maximum activity (1). The incubation was carried out in a Dubnoff shaking incubator[4] (90 min. at 38° C, in the presence of air).

At the end of the incubation period, the reaction was stopped by the addition of 0.1 volume of 3 *M* acetate buffer (pH 5). Each incubate was diluted to 3–5 ml. with water, and extracted three times with 20–30 ml. portions of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate, filtered and evaporated almost to dryness *in vacuo* on a steam bath, then transferred with chloroform to 12 ml. centrifuge tubes and evaporated to a small volume at approximately 70° C, under a stream of nitrogen. The extracts were chromatographed on a 2-cm. paper strip (5), in the ligroin propylene glycol system of Savard (8), for 16–18 hours. The steroid zones were located by application of the Zimmermann reaction (8) to 2-mm. strips cut from each edge of each 2-cm. strip. Δ^4-Androstene-3,17-dione in the remainder of the strip was eluted with 10 ml. of 95% ethanol and suitable aliquots were taken for quantitative determination by the Zimmermann color reaction (9). After correction for the tissue blank, the total amount of product was plotted against the logarithm of the tissue concentration (mg N/1.5 ml.)

EXPERIMENTAL

Recovery of Δ^4-androstene-3,17-dione. Δ^4-Androstene-3,17-dione (100 to 800 μg.) in 0.06 ml. of propylene glycol was added to 1.5 ml. of homogenates containing 0.5 to 13 mg. of N. The reaction was stopped immediately and the steroid extracted. Mean percentage recovery was $63 \pm 3\%$ for 16 trials. Since recovery was consistent regardless of amount of tissue or amount of steroid, the figures reported are not corrected.

Method of Homogenizing. Figure 1 shows curves of activity for two homogenates made from aliquots of the same glands, with the VirTis homogenizer (V) and with the Potter-Elvehjem homogenizer (P). The two curves are identical within the limits of experimental error. Because adrenal tissue is fibrous and homogenization in the Potter-Elvehjem apparatus is therefore difficult, the VirTis apparatus is more suited to large amounts (20–35 gm.) and the Potter-Elvehjem to smaller amounts of tissue.

Figure 1 demonstrates that unfractionated homogenates of adrenal tissue give a reasonably linear relation when the amount of product is plotted against the logarithm of the tissue concentration over the range from approximately 1 to 10 mg. of N.

Influence of Substrate Concentration. It was suggested[5] that the slope of the curve might be influenced, especially at the lower tissue concentrations, by the proportionally greater excess of substrate as the tissue concentrations decreased. Some steroids inhibit *in vitro* enzyme reactions at high concentrations (e.g., the inhibition of d-amino acid oxidase by deoxycorticosterone). Therefore, in one experiment 0.125 gm. of tissue was incubated

[4] Dubnoff shaking incubator available from Precision Scientific Co., Chicago, Ill.

[5] Dr. F. W. Kahnt, Basel, Switzerland, Private Communication.

with 620 and 310 μg. of substrate, 0.06 gm. with 620, 310, and 155 μg., and 0.03 gm. with 620, 310, and 155 μg. At each tissue level, the amount of steroid oxidized was independent of the substrate concentration.

Reproducibility of the Assay Procedure. Multiple assays were performed with an acetone powder of fresh cow adrenal glands, with two to four determinations at each of several concentrations. The data (Fig. 2) give an

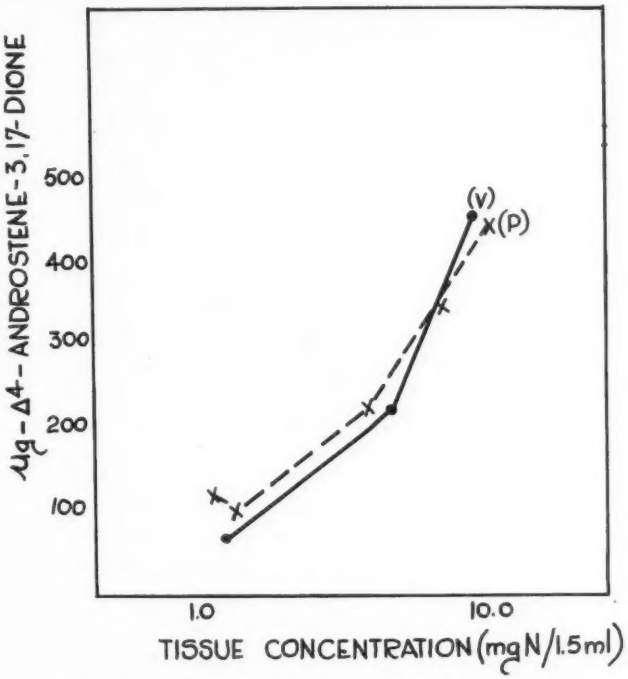

FIG. 1. A comparison of the activities of Potter-Elvehjem and VirTis Homogenates of the same cow adrenal tissue.

indication of the over-all spread. The log tissue concentration-amount of product curves were then calculated by the method of Fisher (10) and plotted (Fig. 3) for (A) all the data shown in Figure 2 (Composite Curve), for (B) a single experiment (T-104), whose data are included in the composite curve as well, and for (C) another experiment (T-109) performed at a later time. The curves are parallel and there is less than 20% deviation of each single assay from the composite curve.

3β-ol-Dehydrogenase Activity of Cow Adrenal Homogenates. A linear relation was found between log tissue concentration and the amount of product (Fig. 4) over the range from 1 to 10 mg. N. Expressed as μg. product per mg. N., the activities ranged from 38 to 110, with a mean of 71 ±9 μg.

Activity of 3β-ol-Dehydrogenase in Cow Adrenals After Storage. Assays were made of aliquots from a pool of adrenal glands when fresh, when frozen for only a few moments, and after being frozen for 1, 8, and 17 days. There was no significant diminution of activity until the glands had been stored in the frozen state for 17 days (30% loss).

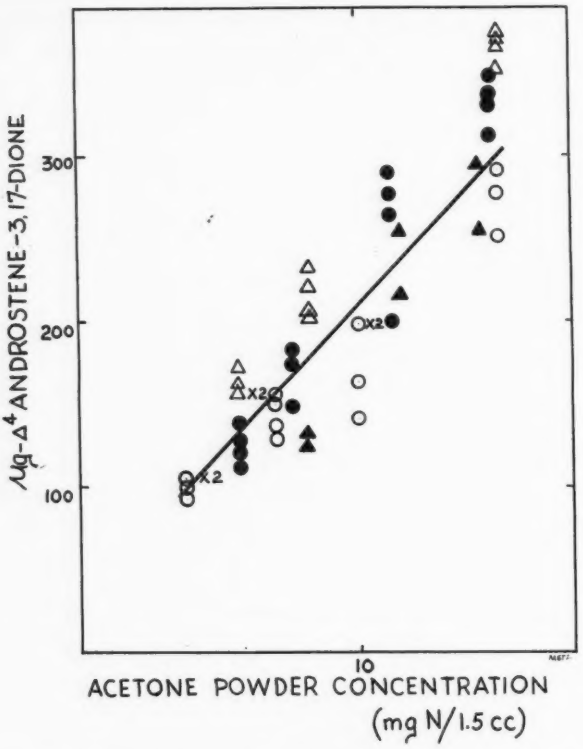

FIG. 2. 3β-ol-Dehydrogenase activity of an acetone powder of cow adrenal glands. Composite curve.

The acetone powder was found to be just as active and possibly more active after being stored for 18 months at −15° C, as when freshly made.

3β-ol-Dehydrogenase Activity of Normal Adrenals from Various Species. Adrenal homogenates of other species also gave a straight line response. Guinea pig and rat adrenals were assayed over the range 0.25–4 mg. N. The activities of these species may be compared by calculating the amount of product obtained per mg. N incubated, since the curves for the various species were parallel. The data are listed in Table 1. Pig adrenals are about half as active as cow adrenals. A normal human adrenal, removed from a

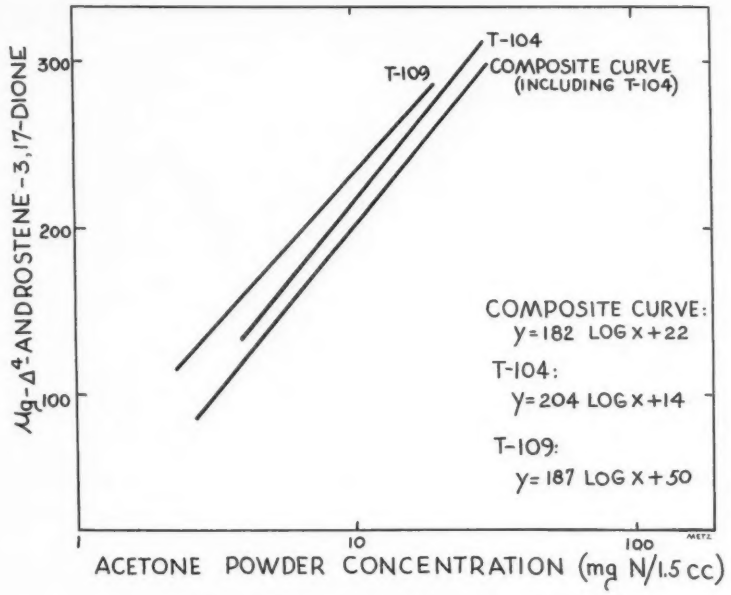

FIG. 3. Comparison of two individual assays of acetone powder with the composite curve.

patient with metastatic breast carcinoma, was about one-fourth as active as cow adrenals. Guinea pig and rat adrenals have approximately 2–4 times as much 3β-ol-dehydrogenase activity as cows.

3β-ol-Dehydrogenase Activity of Adrenals of Guinea Pigs Under Various Experimental Conditions. Because of the great activity and comparatively large size of guinea pig adrenals, it is feasible to assay the activity in a pool

TABLE 1. ADRENAL 3β-OL-DEHYDROGENASE ACTIVITY OF VARIOUS SPECIES—NORMAL TISSUES

Species	Sex	No. of days frozen	No. of assays	No. of assay points	Calculated μg. Δ⁴-androstene-3,17-dione obtained per mg. N (Mean and range of values)
Cattle	F	0–8	7	2–3	71 (38–110)
	M	1	1	4	50
Human	F	10	1	1	21
Rat	M	1	3	2–5	320 (250–460)
Pig	?	0	1	5	44
Guinea pig	M	0	5	4	177 (155–220)

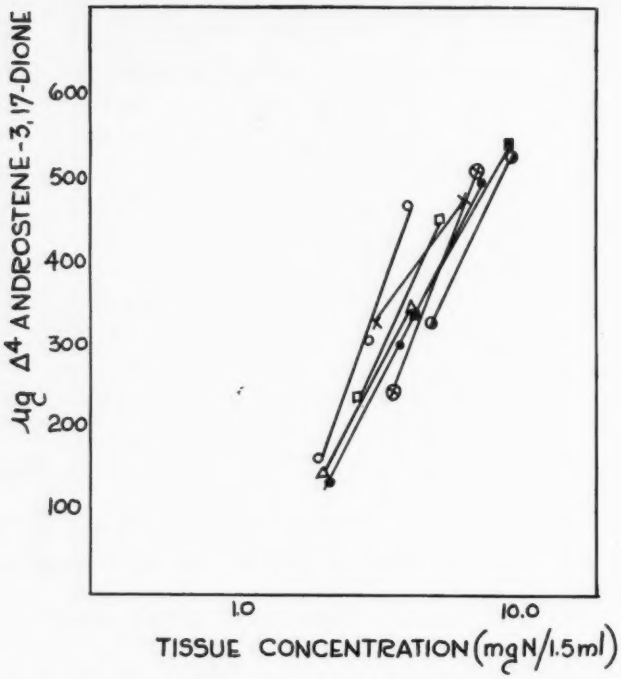

FIG. 4. 3β-ol-Dehydrogenase activity of cow adrenal homogenates.

of adrenals from 5 to 8 guinea pigs. Therefore, they were used to study the effects on 3β-ol-dehydrogenase of various procedures known to influence adrenal corticoid secretion. The results are listed in Table 2. The amount of product per mg. N is listed, the average weight of a pair of adrenals, and an "Index of Total Activity per Animal," which is the product of the above two quantities, and is valid if the assumption is made that an increase in weight of the adrenals reflects an increase in tissue, and not in water only. There are two types of experiments listed. In the first type, conditions known to increase the corticoid output of adrenals were employed. Administration of adrenocorticotropic hormone (ACTH) (1 I.U. per day for 4 days or 20 I.U. per day for 5 days) did not change the concentration of the enzyme on the basis of activity per mg. N. There was some increase in the Index of Total Activity with the larger dose of ACTH, but the total activity was not increased beyond the range of normal control values in other experiments. The work of Li (11) and of Stacke-Dunne (12) suggested that a combination of ACTH with beef growth hormone (STH)[6]

[6] The STH was generously provided by Dr. Sanford Steelman of the Armour Company.

TABLE 2. 3β-ol-DEHYDROGENASE ACTIVITY OF POOLED MALE GUINEA PIG ADRENALS

(5–8 animals per pool)

Conditions	Aver. wt. of pair of adrenals, mg.	Calculated μg. Δ^4-androstene-3,17-dione obtained per mg. N	Index of total activity per animal (μg. product × wt. of 2 adrenals)
Control	580	180	104,500
	360	200	72,000
	316	160	50,500
Control	227	150	34,200
ACTH* (4×1 I.U.)	258	145	37,400
Control	316	155	49,000
ACTH (5×20 I.U.)	479	140	67,000
Irradiated			
c. 2000 r, assayed p 4 days	298	195	58,000
c. 4000 r, assayed p 2 days	331	160	53,000
c. 4000 r, assayed p 4 days	221	175	38,600
Cortisol 10×5 mg.	325	175	57,000
ACTH (4×20 I.U.) +STH† (4×3.5 mg.)	417	150	63,000

* Acthar Gel.
† Armour Beef STH #732-181.

might be more effective, since this combination seems to be necessary to completely restore the adrenals of hypophysectomized rats to normal. However, under the conditions of our experiment (20 I.U. ACTH per day for 4 days and 3.5 mg. STH per day for 4 days), no change in either concentration or total amount of 3β-ol-dehydrogenase activity could be obtained. Irradiation of the guinea pigs with a Cobalt irradiator also had no significant effect on either concentration or total amount of enzyme.

Since hypophysectomized guinea pigs were unavailable, we attempted to suppress the hypophysis by administration of 5 mg. of cortisol per day for 10 days. This treatment was not sufficient to change the concentration of 3β-ol-dehydrogenase or to effect a significant change in adrenal size.

Adrenal 3β-ol-dehydrogenase Activity of Various Human Abnormal Adrenal Tissues. Nine human abnormal adrenal tissues have been assayed. The results are shown in Table 3, with one assay of human normal tissue for comparison. This normal adrenal had an activity of 21 μg. of product per per mg. N. Three of the abnormal tissues have activities equal to this or slightly greater, whereas the activity is considerably lower in six. Some of the decreased activities may be attributable to long-term storage, but this is not true for patient 2, on whose tissue the assay was performed one month after surgery.

Patient 3, whose tissue was stored only one week, had been receiving cortisone therapy for 14 months prior to adrenalectomy.

TABLE 3. 3β-ol-DEHYDROGENASE ACTIVITY OF VARIOUS HUMAN ADRENAL TUMORS

Patient No.	Sex	Age	Diagnosis	Duration of freezing (months)	Calc. μg. Δ^4-androstene-3,17-dione obtained per mg. N
1	F	41	Normal adrenal, metastatic breast carcinoma	⅓	21
2	M	66	Malignant feminizing adrenal tumor	1	5
3	F	38	Arthritis 14 years Cortisone Rx, tumor	¼	5
4	F	28	Adrenogenital syndrome	1/30	19
5	F	32	Virilizing adrenal tumor	20½	6.5
6	M	10	Cushing's syndrome plus virilism	20½	Only faint traces
7	F	28	Acute Cushing's syndrome	"Many"	Only faint traces
8	M	3	Metastases of adrenal rest tumor of testis	¾	28
9	F	29	Cushing's syndrome	2¼	42
10	M	30	Cushing's syndrome, benign functional adenoma	16	8
11	M	43	Pheochromocytoma	¼	5

DISCUSSION

A quantitative assay of adrenal tissue 3β-ol-dehydrogenase activity is obtained by incubating an excess of dehydroepiandrosterone with several concentrations of adrenal homogenate and plotting the μg. of Δ^4-androstene-3,17-dione obtained against the logarithm of the tissue concentration, expressed as mg. N per incubation beaker. A linear relation is found over the range from approximately 1 to 10 mg. N for cow adrenals, and 0.25–4 mg. N for guinea pig and rat adrenals. From data obtained with an acetone powder preparation, employing three concentrations and multiple incubations, an error range of $\pm 20\%$ was obtained.

The enzyme is not destroyed by freezing, but storing of whole tissue in the frozen state results in a loss of 30% of the activity after approximately three weeks. Frozen acetone powder was stable over a period of 20 months.

The 3β-ol-dehydrogenase activity of adrenals of cow and rats measured by this method agrees with the values obtained by Samuels (2), who also found rat adrenals to be approximately three times as active as beef adrenals. Samuels and Helmreich (13) have shown that rat adrenal and testicular 3β-ol-dehydrogenase activities drop after hypophysectomy in terms of total activity, but not of activity per unit weight. Gonadotropin administration increased the enzyme activity of the testes of hypophysectomized rats, but not of the adrenals. We have not as yet been successful in significantly altering the concentration of 3β-ol-dehydrogenase of guinea pig adrenals by administration of ACTH, ACTH plus STH, or large amounts of cortisol, or by subjecting the animals to stress. The ineffectiveness of STH may be due to a species resistance, such as was shown for monkeys by

Knobil *et al.* (14). Hypophysectomized guinea pigs might prove more sensitive to administration of ACTH than intact animals. One human subject who had been receiving cortisone therapy for 14 months had low 3β-ol-dehydrogenase activity.

Huseby *et al.* (15) assayed the 3β-ol-dehydrogenase activity of successive transplant generations of experimentally produced neoplasms of the adrenal cortex of the mouse and found that the concentration of the enzyme roughly paralleled the masculinizing effect of the transplant. Because of the increased excretion of dehydroepiandrosterone in virilizing adrenal cancer, we would expect a low concentration of 3β-ol-dehydrogenase in such conditions. Such low concentrations were found in patients 5 and 6, but no definite conclusions can be drawn since the tissues were stored for over 20 months in the frozen state. The activity of patient 2 was also lower than that of the normal human adrenal, and it was not stored long enough to lose a significant percentage of activity. Patient 9, with Cushing's syndrome, had somewhat increased 3β-ol-dehydrogenase activity, unlike patients 6, 7, and 10, whose tissues were stored for a much longer time before assay.

SUMMARY

A method is presented for the quantitative assay of the enzyme 3β-ol-dehydrogenase in adrenals. An excess of dehydroepiandrosterone is incubated with several concentrations of whole adrenal homogenate. The quantities (in μg.) of Δ^4-androstene-3,17-dione obtained are plotted against the logarithm of the tissue concentration (mg. N). A linear relation is found over the range from approximately 2 to 10 mg. N for cow adrenals and from 0.25 to 4 mg. N for guinea pig and rat adrenals. Studies using a standard acetone powder indicate that the assay is accurate within ±20%.

The assay method has been applied to tissues from various species of animals, to nine specimens of abnormal adrenal tissue from human subjects, and to adrenals of guinea pigs subjected to various measures known to influence the secretion of adrenal cortical steroids.

Acknowledgment

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ANTAGONISTIC ACTION BY FSH ON OVARIAN STIMULATION PRODUCED BY RAT PITUITARY GONADOTROPHIN

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CERTAIN anterior pituitary preparations have been shown to reduce the response of the ovaries of immature rats to exogenous gonadotrophins. This antagonistic action was first associated with preparations rich in growth hormone (1) but was later demonstrated to be more closely associated with the LH (2, 3, 4) or with the FSH (4, 5). However, these experiments have been contradictory and the mechanism of antagonism is not understood.

With the widespread use of gonadotrophins for therapeutic as well as experimental purposes a better understanding of this phenomenon would appear to be of value. The present study was designed to obtain additional knowledge of factors influencing the antagonistic action of FSH against ovarian stimulation produced by a homogenate of rat anterior pituitary glands (RPH).

METHOD AND MATERIALS

Animals. The albino rats used in this study were pen-bred in our laboratory from Wistar stock. Littermates, 21 to 23 days old, weighing 35 to 45 gm., were distributed equally among the control and experimental groups.

Injection Materials. The RPH was prepared by homogenizing adult male rat anterior pituitary glands in normal saline at a concentration of one gland per 1.2 ml. This preparation was found in a preliminary study (6) to be a good ovarian stimulator but not effective as an antagonist.

The FSH[1] was a partially purified extract of hog pituitary glands which was dissolved in normal saline at concentrations indicated in each experiment. It was the most effective of several preparations which were tested for antagonistic activity (6).

Method. All animals were injected twice a day for three days with 0.2 ml of RPH. The experimental animals were injected in addition with FSH at a volume of 0.2 ml. per injection by routes and at times indicated in each experiment. Control animals were injected with an equal quantity of saline.

Autopsies were performed on the morning of the second day after the last injection at which time the ovaries and uteri were cleaned and weighed on a torsion balance.

RESULTS

Effect of dose level of FSH

In this experiment the FSH was administered intraperitoneally simul-

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[1] Follicle stimulating hormone, K47109R, supplied through the courtesy of Dr. Irby Bunding of the Armour Company.

TABLE 1. EFFECT OF DOSAGE OF FSH INJECTED INTRAPERITONEALLY ON THE RESPONSE OF THE OVARIES AND UTERI OF IMMATURE RATS TO SUBCUTANEOUS INJECTIONS OF RAT PITUITARY HOMOGENATE

Group	Total dose FSH in mg.	No. of rats	Ovarian weight, mg.	P[1]	Uterine weight
I	2.16	5	23±1.3[2]	<0.01	71±3.2[2]
II	0.72	5	19±2.1	<0.01	68±4.9
III	0.24	9	19±1.9	<0.01	67±4.0
IV	0.08	7	18±2.5	<0.01	68±7.0
V	0.027	5	25±2.0	<0.05	102±5.5
VI	0.009	5	36±3.8	>0.05	121±8.7
Controls	—	10	48±6.2		100±8.0

[1] Significance of difference between ovarian weights of experimental groups and the control group.
[2] Standard error of the mean.

taneously with each injection of RPH. The total dose was varied from 0.009 to 2.16 mg. as shown in Table 1. The results indicated that 0.08 to 2.16 mg. of FSH markedly reduced ovarian and uterine responses to the RPH preparation. The 0.027 mg. dose was less effective while the 0.009 mg. dose had no statistically significant effect.

On the basis of these results a standardized dose of 0.24 mg. of FSH was selected for the subsequent experiments. At this dose the FSH preparation, injected alone, does not produce significant ovarian or uterine stimulation (6).

Effect of route of injection of FSH

A total dose of 0.24 mg. of FSH was given simultaneously with each injection of RPH by the routes shown in Table 2. Intravenous injections were given at the confluence of the jugular veins, the intramuscular injec-

TABLE 2. EFFECT OF ROUTE OF INJECTION OF FSH ON THE RESPONSE OF THE OVARIES AND UTERI OF IMMATURE RATS TO SUBCUTANEOUS INJECTIONS OF RAT PITUITARY HOMOGENATE

Route of injection	Treatment	No. of rats	Ovarian wt., mg.	P[1]	Uterine wt., mg.
Intravenous	RPH+FSH	9	16±1.3[2]	<0.01	47±4[2]
	RPH	9	33±3.0		109±5
Intraperitoneal	RPH+FSH	19	20±1.0	<0.01	76±5
	RPH	26	44±1.9		130±4
Intramuscular	RPH+FSH	5	28±2.6	>0.05	95±8
	RPH	5	35±3.9		135±8
Subcutaneous	RPH+FSH	9	32±1.0	<0.01	113±5
	RPH	9	39±1.8		129±5

[1] Significance of difference between ovarian weights of experimental and control groups
[2] Standard error of the mean.

tions in the thigh and the subcutaneous injections at a point remote from the injection site of the RPH.

Ovarian and uterine stimulation was more strongly inhibited in the animals injected intraperitoneally or intravenously with the FSH preparation than in those injected intramuscularly or subcutaneously. It is therefore apparent that the degree of antagonism is influenced by the mode of injection of the FSH.

Effect of number of injections per day of FSH

The FSH was injected intraperitoneally one to 12 times per day but the concentration of the hormone was adjusted so that a total dose of 0.24 mg. was given to each group. The daily injection of FSH was administered simultaneously with the first of the two daily injections of RPH. When more than one injection of the antagonist preparation was given per day they were spaced at equal intervals throughout each 24 hour period with two of the injections given at the same time as the injections of RPH.

The results (Table 3) showed that there was marked antagonistic action when the FSH preparation was administered once or twice per day but the effect was considerably less when it was given 4 or 6 times per day. Injection of FSH 12 times per day completely eliminated its antagonistic action.

The uterine weights were quite variable so that there was not a statistically significant difference between control and experimental animals except when the FSH preparation was given twice per day.

TABLE 3. EFFECT OF NUMBER OF INTRAPERITONEAL INJECTIONS PER DAY OF FSH ON THE RESPONSE OF THE OVARIES AND THE UTERI OF IMMATURE RATS TO SUBCUTANEOUS INJECTIONS OF RAT PITUITARY HOMOGENATE

Treatment	Number injections per day of FSH	No. of rats	Ovarian wt., mg.	P_1	Uterine wt., mg.
RPH+FSH	1	5	24±1.5[2]	<0.01	111±15[2]
RPH	—	5	49±3.1		140±13
RPH+FSH	2	19	20±1.0	<0.01	76± 5
RPH	—	26	44±1.9		130± 4
RPH+FSH	4	6	35±2.6	<0.02	116± 3
RPH	—	4	54±6.9		112±14
RPH+FSH	6	19	38±1.7	<0.01	134± 8
RPH	—	19	46±2.1		135± 8
RPH+FSH	12	4	52±1.9	>0.05	159±16
RPH	—	4	38±7.5		182± 9

[1] Significance of difference between ovarian weights of experimental and control groups.
[2] Standard error of the mean.

TABLE 4. EFFECT OF VARYING THE TIME OF INTRAPERITONEAL INJECTIONS OF FSH IN RELATION TO THE TIME OF SUBCUTANEOUS INJECTIONS OF RAT PITUITARY HOMOGENATE ON THE RESPONSE OF THE OVARIES AND UTERI OF IMMATURE RATS

Timing of injections	Treatment	No. of rats	Ovarian wt., mg.	P[1]	Uterine wt. mg.
Alternately 6-hr. interval	RPH+FSH	4	21±0.9[2]	<0.01	125±11[2]
	RPH	4	42±3.0		139± 7
Consecutively	RPH+FSH	10	45±4.4	>0.05	122± 9
	RPH	8	50±4.7		112± 3
Simultaneously	RPH+FSH	19	20±1.0	<0.01	76± 5
	RPH	26	44±1.9		130± 4

[1] Significance of difference between ovarian weights of experimental and control groups.
[2] Standard error of the mean.

Effect of varying the time of injection of the FSH relative to the time of injection of the RPH

A total dose of 0.24 mg of FSH was injected intraperitoneally alternately with the RPH at 6-hour intervals during the three day injection period and compared with consecutive injections in which the FSH was injected twice a day for three days followed by RPH injections on the 5th, 6th, and 7th days. The results are shown in Table 4 together with data of simultaneous injection of FSH and RPH extracted from Table 2. It appears that the degree of antagonism of ovarian growth was as great when the FSH and the RPH were given alternately as when the injections were given simultaneously. However, when the series of FSH injections preceded the series of RPH injections there was no indication of antagonistic action.

Uterine growth was not noticeably inhibited by the FSH injections in either part of the experiment.

This experiment showed that the FSH and RPH injections did not need to be given simultaneously in order to obtain inhibition of ovarian stimulation. However, the series of injections of the FSH must be closely related in time to the series of injections of the RPH in order for the FSH to cause a reduction in ovarian stimulation.

Effect of hypophysectomy on antagonistic action of FSH

All the animals were hypophysectomized at 20 to 22 days of age. Beginning 24 hours after the operation the experimental animals were injected intraperitoneally with FSH simultaneously with each injection of RPH. At autopsy, the sella turcica of each animal was examined under low magnification for pituitary remnants. Incompletely hypophysectomized animals were not included in the data.

TABLE 5. EFFECT OF INTRAPERITONEAL INJECTIONS OF FSH ON THE RESPONSE OF THE OVARIES AND THE UTERI OF HYPOPHYSECTOMIZED IMMATURE RATS TO SUBCUTANEOUS INJECTIONS OF RAT PITUITARY HOMOGENATE

Treatment	No. of rats	Ovarian wt., mg.	P[1]	Uterine wt., mg.
RPH+FSH	12	14±0.7[2]	<0.01	51±11.3[2]
RPH	14	28±2.9		89± 5.7

[1] Significance of difference between ovarian weights of the experimental and the control group.
[2] Standard error of the mean.

The results (Table 5) showed that there was marked antagonistic action by the FSH preparation although the ovarian weights of both the control and experimental animals were considerably below that found in similarly treated normal animals.

The uterine weights of the experimental animals tended to be lower than those of the control animals but the difference was not statistically significant.

It is apparent that removal of the pituitary gland did not interfere with the antagonistic action of the FSH preparation used in this study.

DISCUSSION

The data in Table 2 show that the inhibition of ovarian stimulation was not dependent upon intraperitoneal injection of the antagonist preparation. This is in contrast to the results obtained by Freud (7) and by Leonard (8). On the other hand our results do support Bischoff's hypothesis that the inhibition of ovarian growth is due to the rapid liberation of the antagonist preparation from the site of injection (5). This is shown by the marked antagonistic action obtained in animals given intravenous or intraperitoneal injections of the FSH preparation, which promotes rapid distribution of the hormone from the site of injection, in contrast to the considerably lesser effect observed in animals injected intramuscularly or subcutaneously.

If the rate of absorption is a crucial factor in producing antagonism it is possible that the failure of some investigators to obtain any inhibitory action when their antagonist preparation was injected subcutaneously may have been caused by too slow dispersion of the hormone from the site of injection. Other preparations, such as the one used in the present study, may have been absorbed rapidly enough to cause a small but significant reduction in growth of the ovaries.

The data in Table 3 further support the theory that rapid absorption of a gonadotrophin is essential in order for such a preparation to act antagonistically. Here it can be seen that multiple daily injections of the FSH preparation, which simulated slow absorption to some degree, reduced and finally eliminated the antagonistic effect. These results confirm and extend those obtained by Bischoff (5).

Some consideration can now be given to the possible mechanism of antagonism by the FSH preparation. Previously in this report it has been stated that the FSH preparation reduced the response of the ovary to RPH. However, two other possibilities must be entertained: 1) that the effect was due to inhibition of the release of gonadotrophin from the animals own pituitary gland or 2) inhibition of the RPH by the formation of an antihormone in response to the FSH.

A pituitary effect may be immediately ruled out since antagonism was noted in hypophysectomized animals (Table 5). Therefore, the point of action must be peripheral and not due to an influence on the quantity or quality of the gonadotrophin secreted by the animal's own pituitary gland.

It is not conceivable that a peripheral effect could be brought about by the formation of an antihormone since the experiments were terminated too soon after the injection of the FSH. Furthermore, when the FSH injections preceded the RPH injections (Table 4) which allowed more time for the formation of an antihormone there was no antagonistic effect. Therefore, it seems most likely that antagonism was the result of a decrease in responsiveness of the ovary.

It would be premature to discuss the possible means by which the responsiveness of the ovary may have been influenced but certain characteristics of this inhibitory effect may be pointed out.

It was apparent that the reduced sensitivity of the ovary to RPH was of short duration since the antagonistic effect was completely lost when the series of FSH injections preceded the RPH injections. These results differed from those obtained by Freud (9) who was able to demonstrate antagonism which persisted for 8 days.

There was also some evidence that it was easier to inhibit ovarian growth than uterine growth. This is shown in Tables 1, 3, and 4 in which there are several examples of inhibition of ovarian growth without corresponding inhibition of uterine growth while the reverse was never obtained. This effect can probably be best explained by reference to the work of Heller, Lauson, and Severinghaus (10) who found that extracts of rat hypophyses were able to stimulate the uterus at a much lower dose level than that required to stimulate the ovaries. Therefore, it seems reasonable that it would be easier to inhibit ovarian growth than to reduce the output of the ovarian hormones which stimulate uterine growth.

It is evident from the results of the present investigations that the FSH preparation inhibited the response of the ovaries to RPH and that the degree of inhibition can be influenced by altering the dose, route, number, or timing of the FSH injections but since the FSH preparation was contaminated with LH[2] it may be argued that the antagonistic effect was due to this contaminant However, it can be pointed out that Fraenkel-Conrat

[2] Personal communication from Dr. Irby Bunding.

and coworkers (4) found that purified FSH but not purified LH could act as an antagonist in hypophysectomized rats. We were able to demonstrate antagonism in hypophysectomized animals so that it seems unlikely that LH was responsible for the effect.

Further indication that FSH was the active agent is shown by the fact that the degree of LH contamination was only 5%.[2] Since the minimum effective antagonistic dose of purified LH is 0.005 to 0.01 mg. (4) it is doubtful that there was sufficient LH in the lower doses (Table 1) to inhibit ovarian growth.

On the basis of this evidence it seems likely that FSH was the antagonistic agent in the preparation used in this study.

SUMMARY

A follicle stimulating hormone (FSH) preparation injected intraperitoneally or intravenously twice a day for three days was found to greatly reduce the response of the ovaries of immature rats to a homogenate of adult male rat anterior pituitary glands (RPH) administered subcutaneously. This antagonistic effect on the ovarian response to RPH was greatly reduced or eliminated by injecting the FSH subcutaneously or intramuscularly, or by giving multiple daily injections of the FSH over the three day period. These results were interpreted to indicate that the antagonistic action was dependent on rapid absorption of the FSH preparation into the bloodstream.

The antagonistic effect was demonstrable in hypophysectomized as well as in normal animals which showed that this effect was not the result of a reduction in the output of gonadotrophin by the animal's own pituitary gland. Evidence was presented which indicated that the antagonist exerted its action by reducing the responsiveness of the ovary to the gonadotrophin in the RPH.

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DECREASED INSULIN REQUIREMENT FOLLOWING GROWTH HORMONE ADMINISTRATION IN DIABETIC DOGS[1]

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IT HAS long been known that during injections of diabetogenic anterior pituitary extracts the severity of diabetes in metahypophyseal-diabetic and depancreatized dogs is increased (1); but it is not so well established that subsequently the insulin requirements of these dogs are decreased to less than the initial levels for considerable periods of time (2). These effects are now shown to be exerted by purified growth hormone. The influences of repeated treatments with growth hormone and of variations in the insulin dosage have been studied, and the effects of deprivation of insulin and of adrenocorticotropic hormone (ACTH) have been compared to the effects produced by the growth hormone. Physiological implications of the results have already been indicated (3).

METHODS AND MATERIALS

The methods used for the analysis of blood and urine have been described previously (4). The crystalline bovine growth hormone (Campbell and Davidson, 5) was apparently homogeneous by ultracentrifugal and electrophoretic analysis, thyroid-stimulating hormone could not be detected and contamination by ACTH, prolactin and gonadotrophins was slight to negligible. The growth hormone supplied by the Connaught Medical Research Laboratories (GH, CMRL) was similar. These preparations, dissolved in sterile saline at pH 8, were given subcutaneously twice daily before the meals. Growth hormone prepared by the method of Raben and Westermeyer (6) from pig pituitary glands was kindly donated by Dr. Raben. This less soluble material was made up in a more alkaline medium and was given intraperitoneally. A preparation of ACTH, Lot K9-10-11, from porcine glands of activity 1.5 International Units per mg., was donated by the Connaught Medical Research Laboratories. Ordinary Insulin-Toronto, 40 units per ml., prepared from zinc-insulin crystals was used.

EXPERIMENTAL PROCEDURES AND RESULTS

Permanent pituitary diabetic dogs

Permanent diabetes was induced in four adult male dogs by injections of 3 to 3.5 mg. of purified growth hormone subcutaneously for 15 to 37 days. On ceasing these injections the hyperglycemia (200 to 350 mg.%) was permanent, the urine volumes (1.2 to 1.6 liters) were high, large amounts of sugar (62 to 71 gm.) were excreted daily, and the body weights

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tended to decline despite increased food supply. Insulin was withheld for sufficiently long times to establish the existence of permanent diabetes—in dogs Nos. 14, 15, 83 and 122 these periods lasting 148, 105, 18 and 30 days, respectively. The administration of insulin regularly twice daily was then instituted, and resulted in rapid gains in body weight, enhanced vigor and great zest for food.

The insulin requirement was determined, under standard conditions,

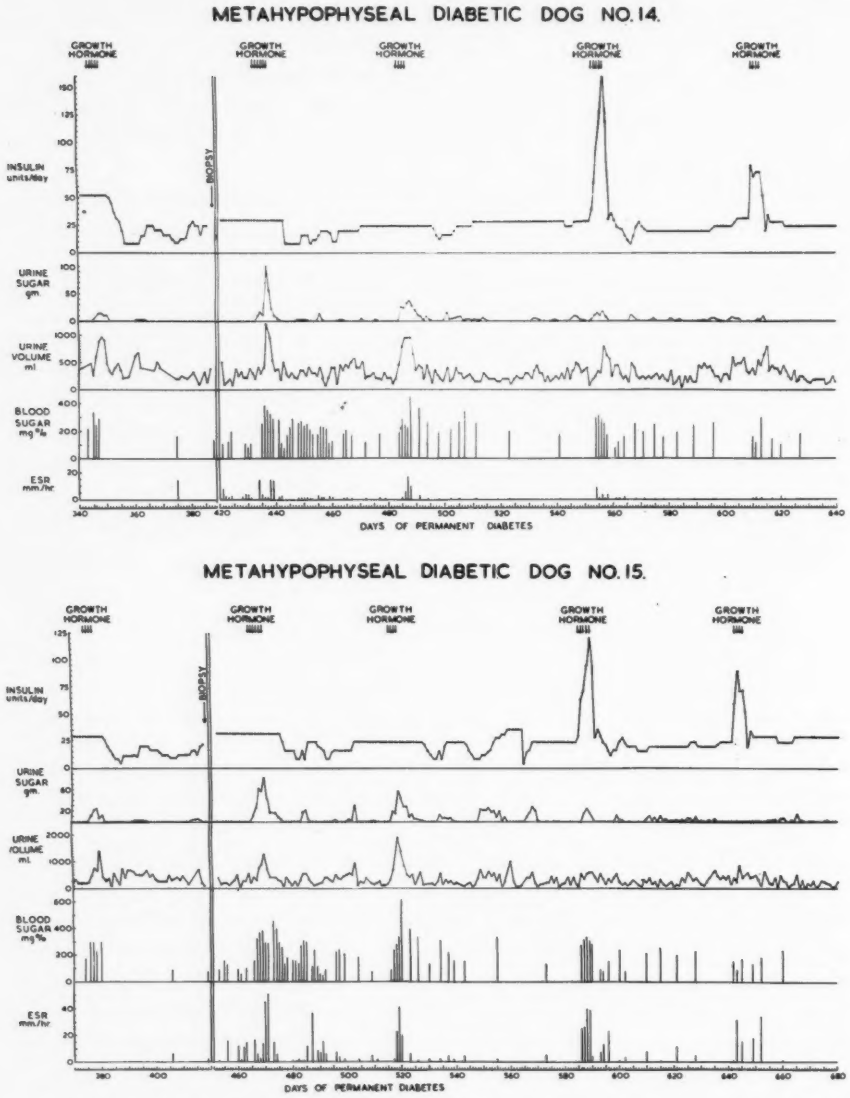
TABLE 1. IMMEDIATE AND DELAYED EFFECTS OF GROWTH HORMONE ON METAHYPOPHYSEAL-DIABETIC DOGS

Observations were taken before, during and after the injection of the metahypophyseal-diabetic dogs with growth hormone (GH), 0.25 mg. per kg. of body weight per day, for periods of 5 to 6 days. Insulin was given during these treatments either in the usual maintenance (M) doses or in increased (I) dosage. The mean sugar excretions for 10 days prior to injection, and during the injections of growth hormone, and the mean increases in blood sugar, ESR and plasma volumes per cent during the treatment over the preinjection values are given. The progression of the changes in the insulin requirements subsequent to the growth hormone treatments are indicated by the arrows.

Dog No.	Duration of metahypophyseal diabetes, days	Growth hormone			Insulin dosage during GH injection	Mean sugar excretion gm./day		Mean increases during GH injections, in blood			Insulin, mean requirements		
		Treatment No.	Preparation No.	Days injected		Before GH	During GH	Sugar, mg.%	ESR mm. per hr.	Plasma vol.%	Before GH units/day	After GH units/day	Change %
14	345	1	R.&.W.	5	M	0	16				52	8→24	−85→−54
	390	Biopsy of pancreas											
	433	2	D25KP	6	M	0	35	225	6	5	32	8→24	−75→−25
	484	3	CMRL	4	M	0	30	105	7	7	24	16→28	−33→+17
	555	4	D34KP	5	I	3	13	75	2	2	28	16→32	−29→+14
	610	5	CMRL	4	I	3	5	41	0	0	32	24	−25
15	375	1	R.&.W.	4	M	0	14	120			28	13	−54
	420	Biopsy of pancreas											
	465	2	D25KP	6	M	0	47	215	11	3	32	16→24	−50→−25
	516	3	CMRL	4	M	0	33	265	25	8	24	8→24	−67→ 0
	585	4	D34KP	5	I	0	13	150	24	2	24	16→24	−33→ 0
	642	5	CMRL	4	I	2	1	0	23	0	24	28→24	+17→ 0
83	53	1	D34KP	5	M	0	46	40	11		36	16→24	−56→−33
	110	2	CMRL	4	I	6	18		0	0	24	24→28	0→+14
122	52	1	CMRL	4	I	2	7	146	24	4	28	24→20	−14→−29

as the daily amount of ordinary insulin, given twice daily with meals, necessary to keep the urine free of sugar or, in some more refractory dogs, to permit the excretion of about 3 gm. of sugar daily. The diet consisted of a moist, prepared dog food composed of meats, grains, starches and minerals, and containing approximately 139 calories, 12–15 gm. of protein and 4–7 gm. of fat per 100 gm. This food was given in the amount of 70 calories per kg. of body weight per day, which was sufficient to maintain body weight.

When the body weights had returned to approximately the initial levels, and the insulin requirements had remained steady for considerable periods, growth hormone was given subcutaneously (0.25 mg. per kg. of body weight per day, as two injections) for periods of 4 to 6 days, while during this time the dosage of insulin was maintained constant or was increased. In the intervals between repetitions of these treatments the insulin requirements were determined with the results summarized in Table 1, and illustrated, for dogs Nos. 14 and 15, in Figures 1 and 2.



FIGS. 1 and 2. The influence of repeated treatments with growth hormone (0.25 mg. per kg. of body weight per day, for 4 to 6 days) on the insulin requirements, etc., of metahypophyseal-diabetic dogs Nos. 14 and 15.

As was expected from previous studies (7), within a day of the first injection of growth hormone increases occurred in the fasting blood sugar, the urine volume and the sugar excretion. These changes persisted during the injection period, but disappeared in 2–3 days thereafter. When the dosage of insulin was increased to 2–5 times the usual during the periods of administration of growth hormone these changes were reduced but not eliminated. The growth hormone also elicited ketonuria (suppressed by the increased doses of insulin); albuminuria of 0.5 to 1 gm. daily in dogs

TABLE 2. IMMEDIATE AND DELAYED EFFECTS OF GROWTH HORMONE ON DEPANCREATIZED DOGS

Observations were taken before, during and after the injection of the depancreatized dogs with growth hormone (GH), 0.25 mg. per kg. of body weight per day for periods of 3 to 5 days. Insulin was given during these treatments either in the usual maintenance (M), or in increased (I) dosage. The mean sugar excretions for 10 days prior to injection, and during the injections of growth hormone, and the mean increases in blood sugar, ESR and plasma volumes per cent during the treatment over the preinjection values are shown. The progressions of the changes in the insulin requirements subsequent to the growth hormone treatments are indicated by the arrows.

Dog No.	Duration of diabetes, days	Growth hormone			Insulin dosage during GH injection	Mean sugar excretion, gm./day		Mean increases during GH injections, in blood			Insulin, mean requirements		
		Treatment No.	Preparation No.	Days injected		Before GH	During GH	Sugar, mg.%	ESR mm. per hr.	Plasma vol.%	Before GH units/day	After GH units/day	Change %
23	20	1	D23KP	4	M	0	51	118	12	3	20	8	−60
	71	2	CMRL	4	M	0	35	76	21	3	8	8	0
	92	3	CMRL	4	M	0	4	76	0	0	8	8→12	0→+50
	123	4	D34KP	5	I	2	30	48	9	2	12	12	0
	179	5	CMRL	4	I	2	0		0	0	12	16	33
25	39	1	D34KP	5	M	0	16	168	4	0	24	12→16	−50→−33
	96	2	CMRL	4	I	4	13	60	0	4	16	20	25
94	75	1	CMRL	4	M	3	75	20	6	2	52	36	−31
	102	2	D34KP	5	I	2	55	177	20	3	36	28	−22
	159	3	CMRL	4	I	4	26	90	0	0	28	32	14
110	51	1	CMRL	3	M	6	72	0	21	2	52	44	−15
111	46	1	CMRL	4	M	3	52	50	3	0	36	28	−22
	80	2	D34KP	5	I	4	36	70	8	4	28	20→28	−29→ 0
	137	3	CMRL	4	I	3	17	100	4	0	28	36	29

Nos. 14 and 15 (also suppressed by increased insulin); and increased erythrocyte sedimentation rate of the blood during most of these treatments, but this effect was apparently not influenced by the dosage of insulin. The maximum increases in these parameters were in most instances considerably greater than the mean increases shown in Tables 1 and 2.

In the metasomatotrophin-diabetic dogs Nos. 14, 15 and 83, subsequent to the first treatment with growth hormone and maintenance doses of insulin, the insulin requirements fell and remained low during 5 weeks, the effect being most pronounced during the first two weeks after the injections. Samples of the pancreas of dogs 14 and 15 were obtained under general anaesthesia 5 weeks after this growth hormone treatment, and the insulin requirement rose after the operation. In metasomatotrophin-diabetic dog 122 the insulin dosage was increased during the first series of

growth hormone injections; subsequently the insulin requirement was reduced, but the effect was relatively small (Table 1).

Following a second treatment with growth hormone the insulin requirement was again reduced in dogs 14 and 15, the lowest level again occurring in the first 1–2 weeks after the treatment and the effect persisting during 6 weeks. During the second treatment in dog 83 the insulin dosage was increased up to 4 times the usual, but following this treatment the insulin requirement did not change during 8 weeks, then increased to 28 units (previously 24).

A third treatment with growth hormone and the usual doses of insulin, had no subsequent effect on the insulin requirement of dog 14. In dog 15 the result was at first uncertain, and complicated by a hypoglycaemic reaction on the 564th day, but the requirement eventually steadied at the same level as before treatment. During the fourth treatment with growth hormone the insulin dosage was increased on successive days up to 6 and 4 times the usual in dogs 14 and 15 respectively. Subsequently the insulin requirement of 14 decreased somewhat during 5 weeks, then increased to slightly above the level prior to the treatment. In dog 15 the requirement also decreased slightly during 4 weeks subsequent to the treatment, then rose to the previous level. After the fifth treatment with growth hormone and increased amounts of insulin a slight and maintained decrease in insulin requirement occurred in 14, but no change in 15.

These experiments show that a consistent decrease in insulin requirement occurred subsequent to treatment of metasomatotrophin-diabetic dogs for a few days with growth hormone. The effect persisted for a few weeks; the duration is uncertain since the periods of decrease were interrupted by new treatments. The effect was most pronounced in dog 14 possibly because its initial insulin requirement was highest. On repetition of the treatment the effect was again obtained, but to a lesser degree, and a third treatment had little or no effect. A larger effect was noted when the insulin given during the growth hormone injections was maintained at the usual level than when it was increased. The experiments also demonstrate the heightened resistance to insulin produced by growth hormone: at the very high insulin dosages the dogs would almost certainly have died if the growth hormone had not been given.

Sections of the samples of the pancreas (about 4 gm.) obtained from metahypophyseal diabetic dogs No. 14 and 15 by biopsy were stained according to Gomori (8), and Wilson (9). The histological examination did not give evidence of the presence of beta cells or beta granulation in the pancreas at a time when the insulin requirements were reduced by the previous administration of growth hormone.

Depancreatized dogs

Of the five adult depancreatized dogs, Nos. 23 and 25 were given 200 gm. of raw pancreas with sufficient prepared dog food to provide 70 calories

per kg. of body weight daily; while Nos. 94, 110 and 111 were given 400 gm. raw minced horse meat, 200 gm. pancreas and 40 gm. sucrose daily, a diet that has been used in previous studies in this Department. The insulin was given with the meals, twice daily.

During the periods of administration of growth hormone, with the maintenance doses of insulin, the urine volume and the sugar excretion increased. Greatly increased doses of insulin failed to decrease the urine volume but the extra sugar excretion was diminished. During the administration of growth hormone ketonuria occurred in most cases, and albuminuria was elicited (except in dog No. 25), but not when the amount of insulin was increased.

Following the first treatment of the five depancreatized dogs with growth hormone, while given the usual amounts of insulin, the insulin requirements decreased (Table 2). However these reductions were not as extensive nor as rapid in onset as in the metasomatotrophin-diabetic dogs. Following the second treatment with growth hormone one depancreatized dog (No. 94) showed a slight decrease in insulin requirement, three showed no change or doubtful effect, while No. 110 succumbed in hypoglycaemia. Following the third treatment the requirement for insulin was not altered in one of the depancreatized dogs, and increased slightly in two of them. After the fourth and fifth treatments of dog No. 25, together with increased doses of insulin, no change in the insulin requirement occurred. Thus the decrease in insulin requirement after growth hormone treatment was not as decided in depancreatized as in metasomatotrophin-diabetic dogs. The effect in the former occurred to a lesser extent, more slowly and on the first occasion only.

EFFECTS OF ADRENOCORTICOTROPHIC HORMONE

Two metahypophyseal diabetic dogs, Nos. 14 and 83, and 4 depancreatized dogs, Nos. 23, 25, 94 and 111, were given ACTH in the daily dosage of 1 mg. per kg. of body weight as two injections, subcutaneously for 4 days, while the usual dosage of insulin was maintained. During the injections the fasting blood sugar, the ESR and the sugar excretion did not increase markedly, nor was any change found in the insulin requirement *subsequent* to these injections.

EFFECTS OF TEMPORARY REDUCTION OF INSULIN

The possibility arose that these effects observed after giving growth hormone might be due to the temporary increases in hyperglycaemia, glycosuria, etc., elicited during the treatment. The insulin requirements of 2 metahypophyseal diabetic dogs and 4 depancreatized dogs were therefore determined under the standard conditions and the insulin dosage was then reduced to half for 8 to 9 days. Marked glycosuria, polydipsia and polyuria resulted. During several weeks following the periods of partial insulin deficiency the insulin requirements were again determined. In one of the

metahypophyseal diabetic dogs (No. 14) the value was the same after, as it was prior to the period of insulin deficiency; and in the other (No. 83) the requirement was slightly increased. In the depancreatized dogs, Nos. 23, 25 and 94, the insulin requirements were the same after as they were before the period of deficiency, and in dog No. 111 the requirement increased from 32 units before to 40 after the treatment. In no case did the temporary insufficiency of insulin decrease the insulin requirements of the diabetic dogs.

DISCUSSION

In metahypophyseal-diabetic dogs the islets of Langerhans are reduced in size and number and the beta cells are especially degenerate (10, 11). The pancreas contains only traces of extractable insulin (12) and its ability to secrete insulin is apparently much reduced or lost (13). The only special treatment required to maintain these dogs in good condition in this laboratory during 5 years has been the administration of insulin. Their metabolic defects can therefore chiefly be attributed to deficiency of insulin, due to failure of secretion of insulin.

There is evidence that growth hormone may stimulate increased secretion of insulin from the pancreas of intact dogs, at least during the early phases of treatment (3, 14). In the rat, growth hormone may cause proliferation of the islets of Langerhans (15), may apparently increase insulin secretion (16) and tends to reduce glycosuria in diabetes due to partial pancreatectomy or alloxan (14, 17). In the cat, recovery from metahypophyseal diabetes occurs, being promoted by treatment with insulin, reduced diet and phlorhizin (18, 19, 20). In the dog, recovery from metahypophyseal diabetes has not been found in this laboratory, nor elsewhere to our knowledge. These observations raise the possibility, however, that growth hormone might promote functional recovery of islet tissue in metahypophyseal diabetic dogs.

The immediate effects of growth hormone in these dogs was intensification of the diabetes. To inhibit this diabetogenic action upwards of 4 times the usual doses of insulin were required. Similar effects were produced in depancreatized dogs. It is obvious that growth hormone creates, through the extra-pancreatic tissues, greatly increased demands for insulin.

Growth hormone was of positive but limited value in reducing the diabetes of metasomatotrophin-diabetic dogs subsequent to administration. A prolonged decrease in insulin requirement occurred after the first treatment, being most marked in the first 2–3 weeks. This response was obtained once again, but not after further treatments. This effect, which is opposite to the immediate effects of growth hormone, must be due to some prolonged alteration in homeostatic adjustments. The causation is obscure. Although the effect was more pronounced in the metasomatotrophin-diabetic than in the depancreatized dogs, the lack of histological

signs of beta cell stimulation or regeneration does not favour the explanation of increased insulin secretion. However caution is required in interpreting this finding, because in the metahypophyseal-diabetic cat disappearance of glycosuria may occur without improvement in the histological characteristics of the beta cells (20). The effect appeared to be more definite when the insulin dosage was maintained constant during the administration of growth hormone than when the insulin dosage was increased temporarily to inhibit the glycosuria, etc. However, exacerbation of the diabetes by temporary reduction of the insulin dosage in diabetic dogs was not effective in decreasing the insulin requirements subsequently. It is possible that these observations may be related to the influence of steroid hormones in reducing the incidence of diabetes in partially-depancreatized rats (21). It might be suggested as a possibility that reduced pituitary secretion might follow the growth hormone treatment.

SUMMARY

In metahypophyseal-diabetic dogs the administration of growth hormone for 4–5 days, with the maintenance doses of insulin, caused the expected increase in the severity of diabetes. *Subsequently* the requirements for insulin decreased relative to the pretreatment values, this effect persisting for weeks. On repetition of the treatments with growth hormone the effect produced was less and finally disappeared. Histological examination of the pancreas during the phase of reduced insulin requirement did not give evidence of regeneration of cells of the islets of Langerhans.

Depancreatized dogs reacted similarly, but reduction of their requirements for insulin following the treatments with growth hormone were less and were apparently slower to develop.

Following temporary increase in the severity of diabetes of metahypophyseal-diabetic and depancreatized dogs, by reduction of the insulin dosage, the insulin requirement was not altered appreciably.

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ENDOCRINE FACTORS DETERMINING THE RATE OF ACCUMULATION OF ENDOMETRIAL SECRETIONS IN EXPERIMENTAL HYDROUTERI OF MICE[1]

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IN A previous report (1) a technique of cervical ligation was described which permits the creation of hydrouteri in rodents and the collection of endometrial secretions (Uterone) in large quantities.

Preliminary information was obtained regarding the effects of some endocrine factors upon the formation of hydrouteri. It was noted that castration delayed the appearance of hydrouteri, that testosterone did not alter this effect of castration and that estrogen given to castrates restored the rate of formation of hydrouteri to that observed in intact animals.

The present paper deals with attempts to determine more precisely the roles of estrogens and androgens and progesterone in the formation and maintenance of hydrouteri.

MATERIALS AND METHODS

Six hundred and sixty Swiss mice of the Tufts colony and ninety-five C57B1/6 mice obtained from the Jackson Memorial Laboratory in Bar Harbor were used. The technique of cervical ligation was the same as described earlier. Presently all animals receive achromycin (Lederle) in their drinking water for a few days following any surgical intervention.

Castration was performed at least five days before uterine ligation, free testosterone and diethylstilbestrol were given as subcutaneous pellets of 50 to 70 mg. of the crystalline material. Whenever histological studies were made, the organs to be sectioned were fixed in 10% formaldehyde and in acid Zenker's solution and processed by standard methods.

The presence of a hydrouterus was ascertained by laparotomy 21, 30, 60, 80 or 110 days after ligation, and its size was measured and graded as follows:

Negative, indicating that no fluid could be aspirated
± when less than 0.1 cc. could be obtained
+ when 0.1 to 0.5 cc. were present
++ with an accumulation of 0.5 to 2 cc.
+++ with 2 to 5 cc. of fluid
++++ when more than 5 cc. were present

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The findings thus obtained could be expressed variously, for instance as follows: how many animals out of a given number showed a + or better hydrouterus after a given time (incidence) how much time had to elapse after ligation before a certain percentage of animals showed a + or better hydrouterus (rate).

The following studies were done:

a) *Magnitude of Technical Error*—In what percentage of animals must we expect failure to produce hydrouteri because the ligature around the cervix is loose or becomes loosened during the days following ligation? Forty Swiss mice were ligated and immediately after the operation placed in cages with mature males of known fertility. Copulation took place in all as shown by plugging of the vagina. In 40 control animals under similar conditions but without cervical ligation, 40 pregnancies occurred. The number of pregnancies occurring in the ligated animals was taken as an indication of the maximal technical failure rate. It may be assumed that this technical error is actually exaggerated since copulation effects a mechanical stress on the cervical ligature not experienced by animals in experiments which excluded mating.

b) *Effect of Stage of Estrous Cycle at Time of Ligation Upon Formation of Hydrouteri*—Vaginal smears of twenty-nine Swiss females were studied by Papanicolaou's exfoliative cytology technique to determine the stage of estrus at the time of cervical ligation. They were accordingly divided into animals in proestrus, estrus, metestrus and diestrus.[2] After 30 days the percentages of animals with + or larger hydrouteri were determined for each group.

In another study, 95 C57B1/6 females were classified at the time of ligation into two groups according to the gross appearance of their uteri. If the uteri were enlarged and turgescent they were classified as "large." If they were pale, thin and atrophic they were listed as "small." Thirty days later the status of the hydrouteri was determined by laparotomy in both groups.

c) *Effect on Hydrouterus Formation of Castration, Estrogens, Testosterone and Progesterone after Cortisone and Estrogen Priming Were Studied in Swiss Mice*—The results of these procedures carried out in groups of from 20 to 40 animals were compared 12 to 30 days after uterine ligation with results obtained in 240 ligated but otherwise intact animals, and were expressed in terms of per cent of animals having + or larger hydrouteri at the time of examination.

d) *Effects of Androgens, Estrogen or Their Combination on the Further Development of Established Hydrouteri*—To study effects of testosterone, 8 Swiss females with well-developed hydrouteri were selected 80 days after ligation. Four of these were intact and four were castrates. All of them received 50–70 mg. testosterone pellets subcutaneously and were laparotomized 7, 14 and 21 days after the insertion of pellets, and the volume of hydrouterine fluid was measured. A similar experiment was carried out giving pellets containing $\frac{1}{6}$ diethylstilbestrol and $\frac{5}{6}$ testosterone. Some of the animals receiving testosterone pellets were also given 0.62 mg. of cortisone acetate suspension subcutaneously for each of four days following pellet implantation.

Progesterone was given in one series in small doses of 1γ per day to estrogen-primed and unprimed animals for twelve days after ligation. Progesterone in larger doses was tested in the following experiment: Forty Swiss mice 3 months old were given 5γ of estradiol benzoate in sesame oil 24 hours before uterine ligation. In 20 of these pellets of progesterone weighing 50–70 mg. were inserted subcutaneously 24 hours after estrogen priming and simultaneously with uterine ligation. Another 20 Swiss mice 3 months old received similar progesterone pellets at the time of uterine ligation without any other

[2] We are grateful to Mrs. L. Bamford, chief cytologist, for these studies.

medication. Laparotomies were done one month following ligation and again 3 and one-half months after ligation.

RESULTS

The technical error causing failure of hydrouterus formation because of leakage through the incompletely ligated cervix was found to be no greater than 15%, since of the 40 animals exposed to males only 6 became pregnant. Two of these delivered at term indicating complete loosening of the cervical ligature and four died because of inability to deliver fetuses that had reached full term size.

The estrus stage of the animal at the time of ligation has considerable importance in determining the outcome of hydrouterus formation. Figure 1

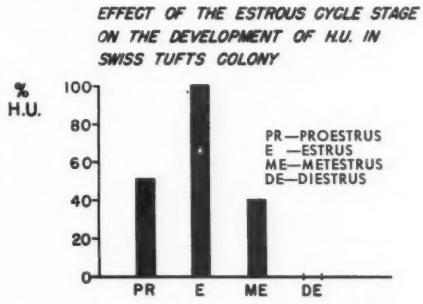

FIG. 1. Effect of the stage of the estrous cycle at the time of cervical ligation on the incidence of hydrouteri formed within one month. (PR signifies proestrus, E—estrus, ME—metestrus and DE—Diestrus.)

shows that all animals which were in estrus at the time of ligation developed hydrouteri whereas only 47% of those in proestrus, 41% of those in metestrus, and none of those in diestrus showed endometrial fluid accumulations.

The importance of the estrus stage for subsequent hydrouterus formation was further emphasized by the observation that 89% of the C57B1/6 mice with large uteri at the time of ligation did develop hydrouteri whereas 85% of those with small uteri at the time of ligation failed to accumulate endometrial fluid.

Several endocrine procedures had significant effects upon the rate of hydrouterus formation as shown in Figure 2. Cholesterol pellets, progesterone administration (in extremely small doses of 1 γ/day) had no significant effects.

One month after ligation in groups of mice treated with larger amounts of progesterone the following rates of positive hydrouteri were observed: estradiol alone 50%, estradiol and progesterone 50%, and progesterone alone 44%. Three and one-half months after ligation the incidence of positive hydrouteri was as follows: estradiol benzoate alone 77%, estradiol and progesterone 94%, and progesterone alone 67%. The hydrouteri of all animals treated with progesterone were distinctly different in appearance from the hydrouteri in untreated, estrogen or testosterone treated animals. The uterine wall appeared much more atrophic and transparent, the fluid

was less cloudy, and fewer cellular elements were found on smears of sediments.

Castration reduced the incidence of hydrouteri as previously seen. Mating caused a slight but significant increase in the incidence of hydrouteri formed, and stilbestrol doubled the incidence normally seen. Testosterone pellets completely prevented hydrouterus formation.

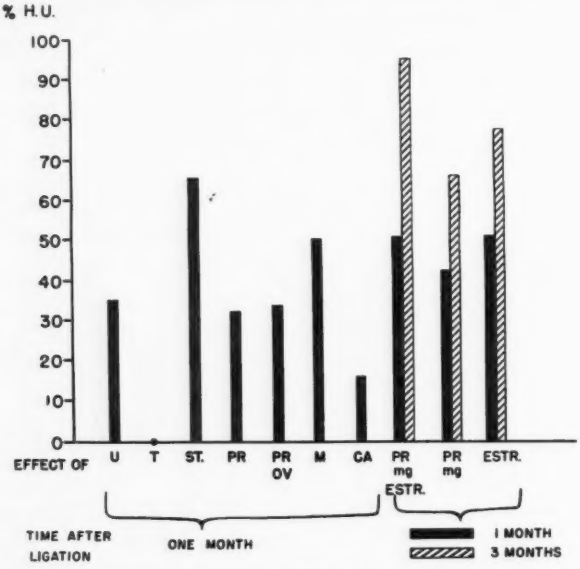

FIG. 2. The influence of endocrine factors of formation of hydrouteri in Swiss mice following ligation of the cervix. The time relationships relative to the day of ligation are show in the abscissa. The letters under each bar indicate the procedure resulting in the percentage of hydrouteri shown by the corresponding bar:

U	uterine ligation without other treatment
T	uterine ligation and testosterone (50–70 mg.) subcutaneous pellets
ST	uterine ligation and stilbestrol (50–70 mg.) subcutaneous pellets
PR	uterine ligation and progesterone, 1γ subcutaneous per day for 12 days after priming with 1γ subcutaneous estradiol benzoate 24 hours preceding ligation
PR OV	uterine ligation same treatment as PR, 5 days after ovariectomy
M	uterine ligation and mating (exposure to male for 30 days immediately following ligation)
CA	uterine ligation 60 days after ovariectomy
PR mg. ESTR	uterine ligation preceded by priming with 5γ subcutaneous estradiol benzoate 24 hrs. before ligation, followed by subcutaneous pellets (50–70 mg.) of progesterone. Laparotomy after one and three months
PR mg.	uterine ligation and progesterone pellets (50–70 mg.) inserted subcutaneously on day of ligation
ESTR	uterine ligation preceded by 5γ s.c. of estradiol benzoate 24 hrs. before ligation

The inhibitory effects of testosterone upon endometrial function were also seen in studies on the effect of 50 to 70 mg. testosterone pellets upon the established hydrouterus. The data are shown in Table 1 and illustrated by photographs in Figure 3.

In intact and ovariectomized animals, the absorption from the pellets of about 1 mg. of free testosterone per day caused marked regression of

TABLE 1

a) EFFECT OF 50–70 MG. OF TESTOSTERONE INSERTED IN SUBCUTANEOUS PELLETS AFTER HYDROUTERI HAD DEVELOPED FULLY

Animal No.	Strain	Time after Uter. lig., days	H.U. size before testoster.	H.U. size of animal with pellets after		
				7 days	14 days	21 days
10	Swiss intact	80	U++++	U+	U±	U−
42	Swiss intact	80	U++++	U±	U±	U−
82	Swiss intact	80	U++++	U+	U±	U−
88	Swiss intact	80	U++++	U+	U±	U−
1	Swiss ovariect.	80	U+++	U+	U−	U−
12	Swiss ovariect.	80	U+++	U+	U±	U−
19	Swiss ovariect,	80	U+++	U+	U±	U−
34	Swiss ovariect.	80	U+++	U+	U±	U−

b) EFFECT OF A COMBINATION OF TESTOSTERONE AND DIETHYLSTILBESTROL GIVEN SUBCUTANEOUSLY IN PELLETS AFTER HYDROUTERI HAD DEVELOPED FULLY

Animal No.	Strain	Time after Uter. lig., days	H.U. size before testoster.	H.U. size of animal with pellets after		
				7 days	14 days	21 days
8	Swiss intact	110	U+++	U++	U++	U++
16	Swiss intact	110	U+	U++	U+++	U+++
31	Swiss intact	110	U+++	U++	U++	U++
34	Swiss intact	110	U+++	U++	U++	U++
4	Swiss ovariect.	110	U++	U±	U±	U±
13	Swiss ovariect.	110	U++	U±	U±	U±
20	Swiss ovariect.	110	U+++	U±	U±	U±
36	Swiss ovariect.	110	U+	U±	U±	U±

the hydrouterus in 7 days and its complete disappearance within 21 days.

In intact animals, this testosterone effect was abolished by 1/5 of the amount of stilbestrol (in mg.) but in the ovariectomized animals, this amount of estrogen did not prevent the inhibiting action of testosterone.

The histologic effect of testosterone consists of a flattening of the endometrium and marked edema in the uterine stroma. The edema fluid contains much periodic acid Schiff positive material. An additional effect of testosterone is the production of uterine tumors probably representing modified deciduomas, in regressing horns of hydrouteri of about half of the animals thus treated. This tumorigenic effect of testosterone in hydrouteri is the subject of another publication (1).

These regressive effects of testosterone on the developed hydrouterus

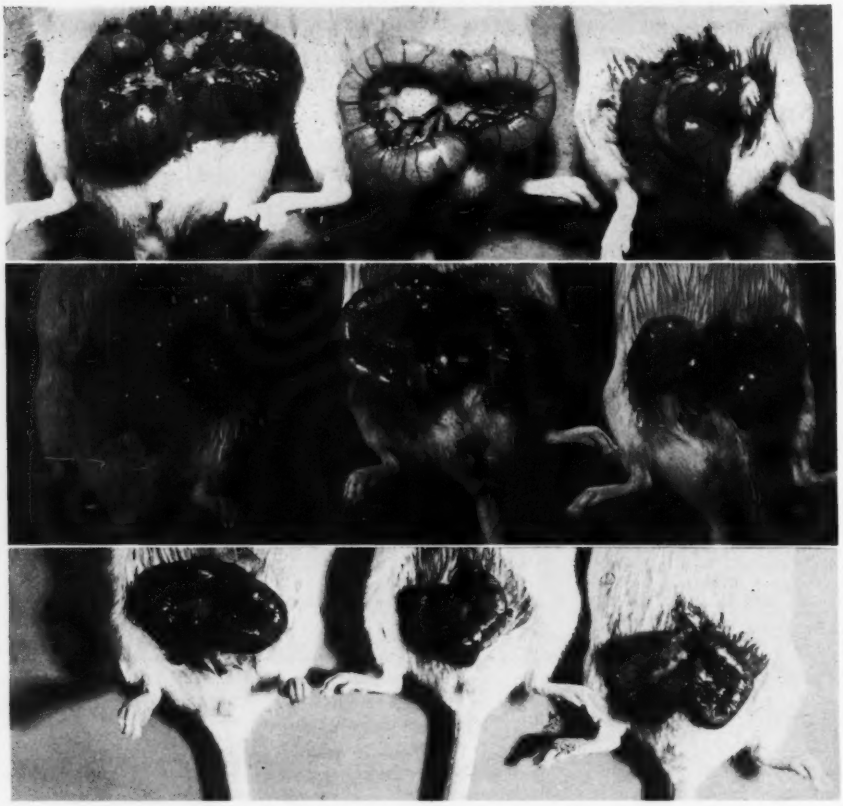

FIG. 3. Illustration of the effects of subcutaneous testosterone pellets on the size of the hydrouterus in Swiss mice—from top to bottom.

a) Untreated mice, one month after ligation of the cervix.
b) The same mice, laparotomized after another month without treatment. Note enlargement of hydrouterus.
c) The same mice one month later and one month after subcutaneous insertion of a 60 mg. pellet of testosterone. Note the marked regression in size of the hydrouterus.

were also greatly diminished by the administration of 0.62 mg. of cortisone acetate suspension given daily for 4 days beginning on the day of the implantation of the testosterone pellets.

DISCUSSION

The ligation of the uterine cervix in mice results in a certain proportion of animals in the accumulation of uterine fluid without any inflammatory change in the uterine wall. The chemical nature of this uterine fluid (2) and its continuous accumulation upon repeated tapping suggest that these are

accumulating endometrial secretions. The histological appearance of the endometrium in such hydrouteri is compatible with secretory activity.

Additional evidence has now been presented showing that the rate of accumulation of hydrouterine fluid is dependent upon endocrine factors such as the stage of estrus at the time of ligation and the presence of estrogens and progesterone. Conversely, large doses of testosterone prevent the accumulation of endometrial secretions. When testosterone is given after a hydrouterus has already been formed, rapid regression of the volume of the accumulated fluid takes place.

This is due to the atrophy of the endometrium and to a change in the structure of the uterine wall which permits effusion of the hydrouterine fluid into and probably through the uterine stroma as shown by the appearance in the submucosa of Schiff periodic acid positive material.

The observations indicating dependence of the rate of accumulation of hydrouterine fluid upon estrogenic and progestational stimulation further support the assumption that the fluid accumulated in experimental hydrouteri represents endometrial secretions. It seems that a high estrogen level at the time of cervical ligation, even though not maintained subsequently, is as effective in increasing the rate of secretion as is the continuous estrogen stimulation.

SUMMARY

Endocrine factors were studied which influence the rate of accumulation of endometrial secretions (uterone) in experimental hydrouteri of mice following ligation of the cervix. Estrus at the time of ligation as well as small amounts of estrogen given at the time of ligation favor the accumulation of uterone as does the administration of estrogen or progesterone (in pellets) during hydrouterus formation. Diestrus at the time of ligation prevents subsequent hydrouterus formation. Testosterone inhibits endometrial secretion when given at the time of cervical ligation and causes regression of hydrouteri once they have been formed. These androgen effects can be prevented by estrogen or cortisone.

Acknowledgments

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BIOLOGIC ACTIVITIES OF ENDOMETRIAL SECRETIONS (UTERONE) COLLECTED FROM EXPERIMENTAL HYDROUTERI OF MICE[1]

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UPON ligation of the cervix uteri in mice, and under appropriate conditions discussed elsewhere (1, 2), a sterile fluid accumulates within the uterus and may be aspirated at suitable intervals resulting in the production of 50 to 200 cc. of such fluid per mouse in the course of a year.

It is postulated that this hydrouterine fluid represents accumulated endometrial secretions and the term UTERONE is proposed for this crude biologic material.

Evidence supporting the assumption that uterone is secreted by the endometrium as well as preliminary data on its chemical composition have been published (1, 2).

There is no information regarding biologic activities of endometrial secretions because no satisfactory methods have been available for the collection of these secretions (3).

The data so far available and to be presented in this paper on biologic activities of murine uterone were all obtained with the crude material used either fresh or following lyophilization and preservation in the cold.

The following effects have been observed following systemic (intraperitoneal) administration of uterone to mice under certain conditions to be specified below.

a) In castrates the size of the uterus is increased by uterone and the size of the adrenals may be decreased.

b) The acceleration of tumor growth of some transplantable murine neoplasms that follows castration and hysterectomy may be counteracted by uterone.

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c) In large doses, uterone produced a fulminating exophthalmos, retro-ocular hemorrhage and necrotizing ophthalmia.

MATERIALS AND METHODS

Mice of various inbred strains of the Jackson Memorial Laboratory and Swiss mice of the Tufts colony were used. The animals were kept in the Summer investigators' laboratory at Bar Harbor for most of these experiments (not air-conditioned) and in the air conditioned Tufts animal rooms (72° F. humidity—65%) for some of these studies. All animals were fed Purina Chow Checkers exclusively, and received achromycin 1 mg./ml./da. in the drinking water for several days after any surgical procedures. Surgical procedures such as castration and tumor transplantation were done by standard methods, and in the case of castration, under nembutal and ether anesthesia.

Uterone was obtained by aspirating hydrouterine fluid at approximately monthly intervals from intact Swiss mice and BALB/C mice. These amounts of hydrouterine fluid were pooled, cultured to ascertain sterility and kept in the icebox for varying lengths of time prior to lyophilization. Later, batches were frozen immediately after pooling and thawed only once prior to lyophilization. The lyophilized powder was kept in a deep freeze. Fraction IV of human serum, obtained by Cohn's method of fractionation was used as a control substance in some of the experiments because of its high mucoprotein content.

The tumors used in transplantation experiments were Sarcoma 180 and Sarcoma 1 obtained from the Jackson Memorial Laboratory. Tumor suspensions were prepared in Ringer's solution by means of the Snell cytosieve and were adjusted to a cell concentration of 2% as measured by a Wintrobe hematocrit tube. Test tubes containing the tumor suspensions were kept in iced water during the time when the injections were made. In every instance, one and the same tumor suspension was used for an entire experiment and the order of animals in which inoculations were made was so arranged that the members of each group in each experiment had received tumor cells of similar suspension age. The viability and rate of growth of the tumor cells at the end of the inoculations was further ascertained by injecting the tumor suspension remaining at the end of the experiment into a separate control group.

At the end of each experiment the animals were killed by cervical dislocation and complete autopsies were made by one and the same person in each experiment. The organs and tumors were preserved in 10% formaldehyde for histologic study.

In those instances where weights were obtained, tumors or organs (uteri, adrenals) were carefully dissected free of fat and weighed immediately (wet weight) and in some instances again after drying to constant weight in an oven (dry weight).

a) *Effects of Uterone on Size of the Uterus and Adrenal.*—In a first series of experiments the effects of uterone on adrenal size and uterine size of intact mice, sham operated castrates and hysterectomized castrates were studied in BALB/C mice which also carried Sarcoma 180. This combined experiment was done because uterone was then available in only small quantities. There were 70 animals in the group receiving Uterone and 71 received saline as control. These groups were further subdivided into 33 intact animals, 13 sham operated animals which underwent laparotomy and manipulations of uterus and ovaries, 39 ovariectomized animals and 37 ovariectomized and hysterectomized animals.

All of the animals received 0.1 ml. of the Sarcoma 180 tumor suspension 3 days after their operations. On the day of transplantation 0.2 ml. of Uterone (or saline in the controls) was given and thereafter for 7 days 0.1 ml. of saline or Uterone respectively were given. The animals were killed on the 13th day, the tumors were weighed and autopsies were performed.

The effects of Uterone on uteri and adrenals of castrated Swiss mice were further studied in 86 mature animals from the Tufts colony. These were divided into groups re-

ceiving 0.2 ml. Uterone intraperitoneally daily for 8 days beginning at least 5 days after castration and two control groups, one receiving saline in similar doses, the other receiving no treatment. These animals were killed and autopsied on the 9th day after the start of the injections.

Finally similar experiments were carried out on 46 immature, intact Swiss mice and at autopsy careful attention was paid to the appearance (gross and microscopic) of the ovaries to detect the presence or absence of gonadotropic hormones in the injected material.

While the preceding experiments were done at a single dosage level of Uterone, a dose response study was made in AKS mice castrated 8 days prior to the first injection of Uterone. The solution of Uterone, obtained from Swiss mice, sterile and lyophilized, was so adjusted by addition of distilled water that 9 groups of 10 animals each received a total dose of 1, 2, 4, 8, 16, 32, 64, 128 and 256 mg. respectively of dry material per mouse over a 7-day period. In this instance Fraction IV of human serum was used in control experiments. The animals were closely observed during this time and killed and autopsied on the 8th. day of the experiment.

b) *Effects of Uterone on the Growth of Transplantable Tumors.*—Experiments on effects of Uterone on Sarcoma 180 in BALB/C mice were done simultaneously with the studies on effects of Uterone on adrenals and uterus and have been described above.

AKS females and Sarcoma 1 were used in another experiment—60 females were castrated and ovariectomized and 2 days later 0.2 cc. of a 2% Ringer's suspension of Sarcoma 1 were injected subcutaneously in all of these and also into 20 untreated control animals. Beginning the next day, the following injection schedules were carried out on these animals:

1) Intact control animals, no injections.
2) 20 hysterectomized and ovariectomized animals 128 mg. (dry substance) of uterine per mouse given in 4 days intraperitoneally.
3) 20 hysterectomized and ovariectomized animals given corresponding amounts of saline for 4 days
4) 20 hysterectomized and ovariectomized animals given 128 mg. (dry substance) of Fraction IV in the same manner as Uterone was used in Group 2.

c) *Destruction of the Eye by Uterone.*—The appearance of acute malignant exophthalmos in some of the animals used in the experiments described above led to separate studies on toxic effects of Uterone.

A total of 32–64 mg. of solid Uterone per mouse was given to the following animals, and the eyes were observed and results recorded: BBF1, 12 females, Swiss R, 12 females, BALB/C hysterectomized and ovariectomized, 7 animals, AKS, ovariectomized, 10 animals each at dosage levels of 32, 48 and 64 mg. AKS, hysterectomized and ovariectomized, 20 animals. The Uterone used in these studies had been obtained from Swiss mice.

RESULTS

Effects of Uterone upon Size of Uterus and Adrenals in Castrates.—In BALB/C mice bearing Sarcoma 180, the adrenals were relatively large, and the administration of Uterone reduced their weight below that of adrenals of normal controls. The uteri of animals receiving Uterone were significantly larger and heavier than those of the controls. These data are illustrated in Figure 1. They were, however, pale in appearance, much like the control uteri of the untreated castrates and showed none of the congestion and turgescence seen in uteri of animals in estrus. Histological examination showed simple myometrial hyperplasia as compared with the controls.

If viewed alone, without knowledge of the other groups these uteri would have been observed as "normal" (Fig. 2).

Table 1 shows that the effect of Uterone upon the uterus of mature castrates is also consistent in Swiss mice and statistically significant in most instances. The same is true for the weight reduction of the adrenals following injection of Uterone.

In immature Swiss mice the changes of uterine weights were in the same

FIG. 1. Changes in uterine and adrenal size of castrates caused by intraperitoneal injection of Uterone from Swiss mice into recipient Swiss mice 3–4 months old.

direction of an increase, though not statistically significant and the adrenals remained unchanged. Neither were there any changes in the gross and histologic appearance of the ovaries in these immature animals, indicating that Uterone does not contain gonadotropins. In castrated AKS mice receiving increasing doses of Uterone in geometrical progression, no effects upon uterine size or weight occurred at dosages below 32 mg. Above that dosage the toxicity of Uterone began to manifest itself and many animals died, reducing the number available for analysis. The uteri of the remaining animals, however, were larger than those of the controls and much like the ones in the earlier experiments in BALB/C mice. Histological examination again revealed simple hyperplasia. The adrenals of these animals were smaller than those in the BALB/C mice and no further reduction in weight followed the administration of Uterone.

Effects of Uterone upon the Growth of Transplantable Tumors.—In BALB/C mice, Sarcoma 180 grew considerably larger in the castrated and hysterectomized animals than in intact controls or sham operated animals.

The injection of Uterone was without effect on tumor growth in any but the hysterectomized castrates. In such animals, when they received Uterone, the growth rate of Sarcoma 180 was reduced to that found in intact animals. The take rate seemed to be significantly higher in animals receiving Uterone. Essentially similar results were obtained in AKS mice bearing Sarcoma 1. In this experiment, hysterectomized castrates receiving saline or Fraction IV had tumors which were significantly larger than those

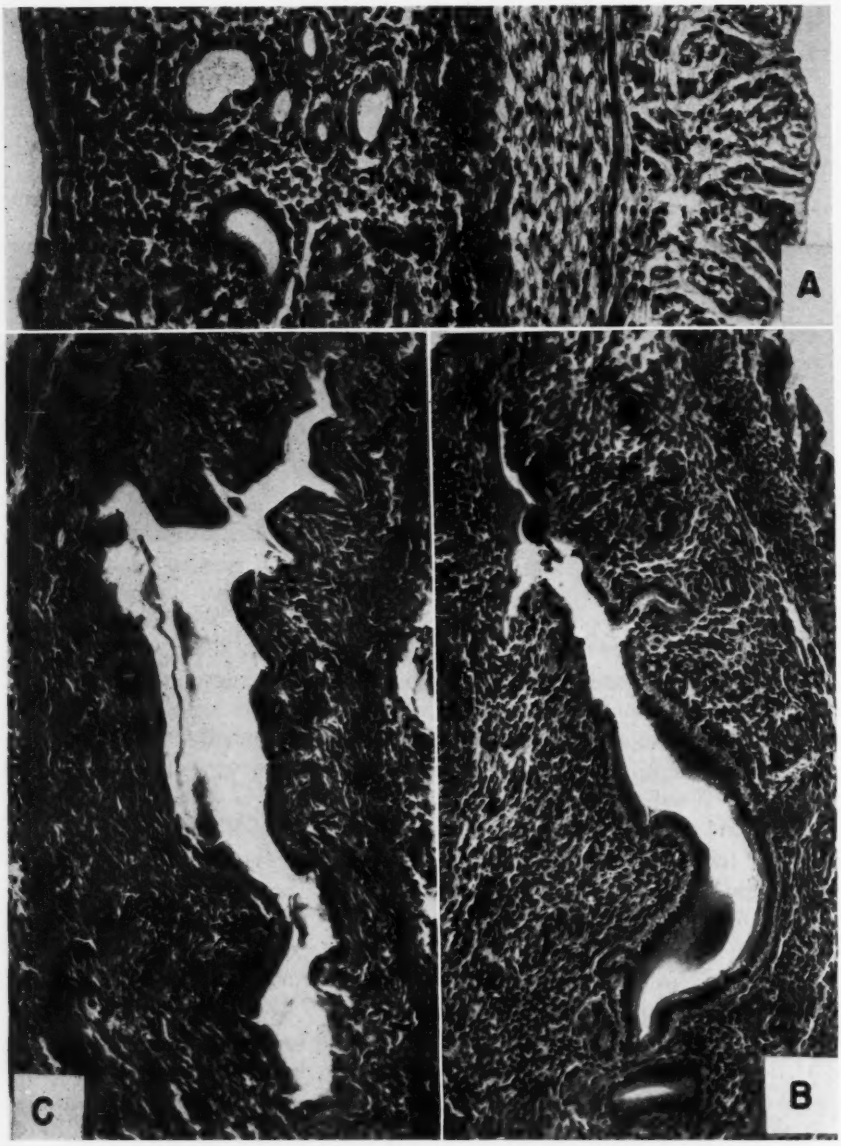

FIG. 2. Comparison of histologic appearance of uteri of intact Swiss mice. (A)—Castrates having received intraperitoneal Uterone (B)—Castrates having received saline injections (C). All preparations are hematoxylin eosin stained; microphotographs ×400. Note that the castration atrophy is less marked in the uterus of the animal having received uterone (B).

obtained in untreated intact controls. Tumors in hysterectomized castrates receiving Uterone grew no larger than those of intact untreated controls (Fig. 3).

The Toxic Effects of Uterone upon the Eye.—When more than 32 mg. of Uterone were given to female mice of various strains about half of them developed acute disorders of the eye. Grossly this appeared to consist of an acute intraocular hemorrhage in a few cases, and most frequently a retro-bulbar hemorrhage with marked exophthalmus, usually, but not

TABLE 1. EFFECT OF UTERONE ON THE WEIGHT OF THE UTERUS AND ADRENALS IN MATURE CASTRATED SWISS MICE, TUFTS COLONY

Group	Treatment	No. animals	Age, mos.	Aver. body weight, gm.	Aver. wet uter. weight, mg.	Aver. dry uter. weight, mg.	Aver. wet adren. weight, mg.	Aver. dry adren. weight, mg.
A	Uterone	20	3–4	26.2	56	14.6	4.8	1.1
B	Saline	20	3–4	26.9	36	8.0	8.4	2.07
C	No treatment	5	3–4	27.2	28.7	7.5	7.4	2.06
D	Uterone	15	2–3	24.4	27.2	—	5.9	—
E	Saline	15	2–3	24.6	19.2	—	8.4	—
F	No treatment	11	2–3	24.5	19.8	—	9.1	—

Statistical evaluation—Mature castrated Swiss mice

	Groups compared	t	P		Groups compared	t	P
Wet weights of uteri	A–B	2.0	0.05–0.1	Wet weights of adrenals	A–B	3.9	less than 0.01
	A–C	2.1	0.05–0.1		A–C	4.2	less than 0.01
	D–E	1.9	0.05–0.1		D–E	2.5	0.02–0.05
	D–F	1.8	0.05–0.1		D–F	2.3	0.20–0.05
Dry weights of uteri	A–B	1.9	0.05–0.1	Dry weights of adrenals	A–B	2.5	0.01–0.02
	A–C	1.5	0.01–0.2		A–C	1.8	0.05–0.1

Effect of uterone on the weight of the uterus and adrenals in immature intact Swiss mice, Tufts Colony

Group	Treatment	No. animals	Age, weeks	Aver. wet uter. weight, mg.	Aver. wet adren. weight, mg.
G	Uterone	12	3	30.0	5.7
H	Saline	10	3	24.0	5.7
I	Uterone	6	3	30.0	5.7
J	Saline	6	3	24.0	5.7

t and P—Not significant.

always, bilateral and leading within a few days to loss of the eye by necrosis and death of the animals (Fig. 4). The incidence of this disorder is shown in Table 2.

The development of this syndrome of "black eye" was so rapid that it was nearly impossible to obtain satisfactory histological sections before eye and socket were extensively necrotic.

In a few animals, "black eye" occurred during absorption of their own hydrouterine fluid either following testosterone administration or after trauma to the hydrouterus.

So far the syndrome could not be produced in Swiss mice by injection of Uterone, but has been seen even in one animal of the Swiss strain upon absorption of endogenous endometrial secretions.

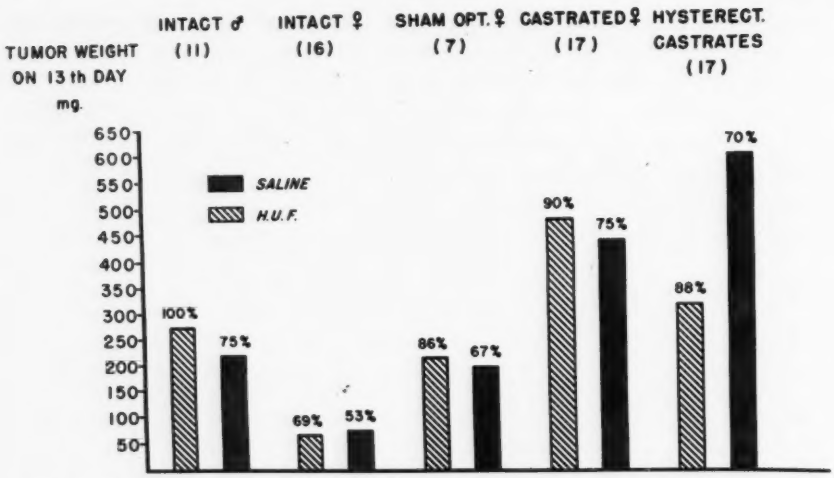

FIG. 3. Effect of endocrine status and lyophilized Uterone from Swiss mice on the subcutaneous growth of Sarcoma 180 in BALB/C mice.

Ovomucoid, crystalline, from the Worthington Biochemical Sales Company Freehold, New Jersey, in doses of 64 and 128 mg intraperitoneally in distilled water, did not produce any "black eye."

DISCUSSION

It appears that endometrial secretions collected from experimental hydrouteri of mice, lyophilized and redissolved in distilled water (Uterone) have certain biologic effects upon the uterus, the adrenal, and the growth

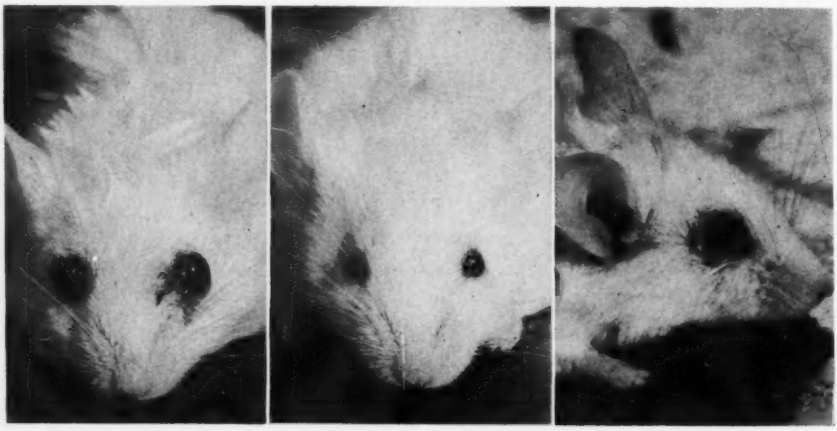

FIG. 4. Various stages of the syndrome of "Black Eye" induced within a few hours by intraperitoneal injection of at least 32 mg. of Uterone.

of transplanted tumors. In addition, they possess properties which produce acute ophthalmic and retrobulbar hemorrhages. These last named toxic properties render more difficult the study of other biologic activities since they manifest themselves at dosage levels where other activities also occur. All of the biologic activities of Uterone so far observed have a certain degree of strain specificity, the uteri of castrates were enlarged only in the BALB/C and AKS and Swiss strain but not in C57BR mice, reduction of adrenal size was found in BALB/C mice (bearing Sarcoma 180) and to a

TABLE 2. THE OCCURRENCE AND INCIDENCE IN VARIOUS STRAINS OF MICE OF THE "BLACK EYE" SYNDROME CAUSED BY UTERONE

Strain	No. animals	Dosage uterone	Eye changes	Time of appearance after inj.
BBF_1	12 ♀	64 mg.	8 (exophthalmos ophthalmitis)	16 hrs.
SWR	12 ♀	64 mg.	no toixcity	
BALB/C, hyster.-ovect.	7 ♀	64 mg.	1 ophthalmitis	24 hrs.
AKS, ovariectomized	10	32 mg.	1 black eye	4 days
	10	48 mg.	6 black eye	2–3 days
	10	64 mg.	9 black eye	2–3 days
AKS, hysterectomized and ovariectomized	20	8–32 mg.	12 black eye	2–3 days

lesser degree in other strains, and the toxic manifestations (the "black eye" syndrome) occurred in BBFl, BALB/C and AKS mice, but not in Swiss mice when Uterone was injected intraperitoneally. It was observed once in a Swiss mouse when following accidental puncture this animal absorbed the endometrial fluid accumulated in its own hydrouterus.

The inhibitory effects on the growth of transplanted tumors were found in Sarcoma 180 growing in BALB/C mice and in Sarcoma 1 growing in AKS mice. No such effects occurred in Sarcoma 180 growing in Swiss or C57BR mice. The growth of these tumors was not inhibited in intact animals but only in those wherein the tumors grew more rapidly following hysterectomy and castration than they did in normal controls. In contrast to its tumor inhibiting effects on the rate of growth Uterone seemed to facilitate the "take" of tumors.

The accelerated growth rate of Sarcoma 180 in BALB/C mice and of Sarcoma 1 in AKS mice also appears to be a strain specific phenomenon since it did not occur in Swiss or C57BR mice. It may be postulated that the uterus possesses a tumor growth inhibiting function in certain strains and that in these strains Uterone may be the hormonal agent that mediates this effect.

On the basis of the published data and of our own observations the work-

ing hypothesis may be postulated that some of the observations on the inhibition of tumor growth by estrogen administration or pregnancy may be due to effects of Uterone rather than the direct action of estrogen.

The production of acute exophthalmus due to retro-ocular hemorrhage and of intraocular hemorrhages ("black eye") by a crude material secreted by an organ of the same species is extremely interesting and as far as we know, unique. The mechanism of this morbid process is still obscure. Except in a single case where there was also a massive hemorrhage in the tongue, no other hemorrhagic manifestations have been seen in these animals, and the disturbance has been localized to the eye and socket.

The effects observed on the uteri of castrates are not like those seen with estrogens or progestational substances but appear to consist mainly of a growth stimulation of the uterine muscle fibers.

Since these studies have all been carried out on crude Uterone, we have so far, no clue as to the nature of the active principles involved in the phenomena which were observed. It may be safely assumed that these are not steroids. C^{14} labeled acetate given to animals during the formation of hydrouterine fluid in doses where labeled steroids would have been recovered from organs secreting them, failed to appear in detectable amounts in hydrouterine fluid.[5]

Studies on fractionating Uterone and on its purification are currently underway.

SUMMARY

Endometrial secretions accumulating in the hydrouteri of mice after ligation of their cervices possessed the following biologic activities: Reduction of adrenal size in castrates; Induction of simple hyperplasia of uterus in castrates; Inhibition of accelerated growth of transplanted tumors in hysterectomized castrates; Production of an acute retrobulbar hemorrhage in the eye sockets of a large proportion of mice receiving more than 32 mg. of dry uterone intraperitoneally.

The name of UTERONE is proposed to describe crude preparations of endometrial secretions obtained by ligation of the cervix uteri.

Acknowledgments

We are indebted to The Protein Foundation, Cambridge, Mass. for generous supplies of Cohn's Fraction IV, and to the Lederle Laboratories Division, American Cyanamid Co. for the Achromycin used in this study.

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[5] We are indebted to Dr. Kenneth Savard of the Worcester Foundation for Biological Research for having done these studies.

DIFFERENCES IN THE MAMMARY RESPONSE TO ESTRONE AND PROGESTERONE IN CASTRATE MALE MICE OF SEVERAL STRAINS AND HYBRIDS

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INASMUCH as an appropriate hormonal milieu is one of the factors essential for mammary tumorigenesis in mice, a number of workers have examined the hypothesis that strain differences in mammary tumor incidence may be related in part to differential mammary sensitivity to estrogen (*cf.* 1). It has been claimed that in castrated male mice of strains highly susceptible to mammary tumor development the duct system of the mammary glands is more responsive to estrogen than in less susceptible strains (2), and also that mammary ductal and alveolar development is more extensive in response to estrogen in strains with high tumor susceptibility (3, 4). Bonser (5) also reported differences in alveolar response to estrogen in strains with high and low tumor susceptibility. Gardner *et al.* (6) reported no difference in ductal response to estrogen, but did find localized atypical alveolar hyperplasia in the high tumor C3H strain. On the other hand, Mühlbock (7) reported that the mammary glands of certain high and low tumor strains were equally sensitive to estrone.

Daane and Lyons (8) have found that daily injections of 2.5 μg. estrone and 1 mg. progesterone into castrated male C3H mice will induce mammary growth similar to that of the first half of pregnancy in this strain. Blair and Daane (9) reported briefly that this combined steroid treatment for short periods produced greater alveolar response and slower ductal development in castrate male mice of the high-tumor C3H/He strain than in those of the low-tumor C57Bl strain. The A/He strain (tumor incidence low in virgin, high in parous mice) showed alveolar and ductal responses intermediate between the C3H and the C57Bl strains.

The present experiment utilizes the appreciable alveolar, as well as ductal, development seen after the combined administration of estrone and progesterone in an investigation of strain differences both in growth rate and in extent of development attained in the mammary glands of castrate male mice.

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[1] Predoctoral Fellow in Biology of the National Science Foundation.

MATERIALS AND METHODS

320 male mice of 4 inbred strains (C3H/HeCRGL, C57Bl/CRGL, A/HeCRGL, and BALB/c/CRGL) and of 2 reciprocal hybrid series of these strains (C3H×C57Bl and A×BALB/c) were used in this study. In addition, 30 male C3H/HeCRGL mice derived from stock freed of the mammary tumor agent by foster-nursing on C57Bl mothers were employed (C3Hd). In designations of hybrids, the female parent is mentioned first.

All animals were bilaterally castrated at 11 weeks of age under nembutal anesthesia

TABLE 1. DEGREE OF MAMMARY ALVEOLAR DEVELOPMENT* IN CASTRATE MALE MICE OF SEVERAL STRAINS AND HYBRIDS AFTER TREATMENT WITH ESTRONE AND PROGESTERONE

Days of hormone treatment	Strains								
	C3Hd	C3H	C3H × C57	C57 × C3H	C57	A	A × BALB/c	BALB/c × A	BALB/c
0 day untreated control	0 (7)	0 (5)	0 (9)	0 (6)	0 (6)	0 (7)	0 (8)	0 (3)	0 (5)
15 days	0 to + (10)	+ to ++ (11)	+ (7)	0 to + (15)	+ (8)	++ (7)	++ (3)	0 to + (4)	+ to ++ (7)
25 days		+ to ++ (3)	+ (6)	0 to + (3)	0 to + (3)	++ (3)	+ to ++ (3)	0 to + (2)	+ to ++ (3)
35 days		++ (3)	+ (3)	+ (2)	0 to + (3)	+ to ++ (3)	+ to ++ (3)	+ (3)	+ to ++ (3)
45 days	+ (3)	++ (3)	+ (3)	+ (3)	+ (3)	+ to ++ (3)	+ to ++ (3)	+ to ++ (2)	+ to ++ (3)
55 days	++ (4)	+++ (3)	+ (3)	+ (3)	+ (3)	++ (3)	++ (3)	++ (3)	++ (3)
65 days	+++ (3)	++++ (3)	+ (6)	+ (3)	++ (3)	++ to +++ (3)	++ (3)	++ (3)	++ to +++ (3)
75 days		+++ to ++++ (3)	+ (2)	++ (3)	++ (3)	++ (2)	++ (3)	++ (3)	++ (4)
85 days	++ (3)	+++ HN (3)	++ HN (4)	++++ (3)	+ (6)	++ (3)	++ (2)	++ (3)	+ (4)
85 day oil-treated control	0 (7)	0 (5)	0 (6)	0 (3)	0 (4)	0 (7)	0 (2)	0 (5)	0 (4)

* As expressed by 0 to ++++ (see text for explanation of symbols). Numbers of animals are given in parentheses. HN = hyperplastic nodules.

(10), and one week later hormone treatment was started. Experimental animals were injected subcutaneously with 5 μg. estrone and 2 mg. progesterone in 0.1 ml. sesame oil every two days. Controls received 0.1 ml. sesame oil. Two to 15 mice of each strain and hybrid (Table 1) were killed 15 days after the start of injections (day 15), and subsequently at 10-day intervals until 85 days. Groups of the C3Hd strain were sacrificed at 15, 45, 65, and 85 days only. Control animals were killed before the onset of hormone treatment (day 0) and at day 85.

At sacrifice, the skin with the adherent mammary glands was removed from each mouse and fixed in 15% formalin. After removal from the skin, all glands were stained with alum-carmine, dehydrated, cleared, and mounted on slides in piccolyte.

After preliminary examination of the general features of mammary gland architecture, the glands of each animal were evaluated as follows:

(1) The degree of lobulo-alveolar development was estimated according to a series of arbitrary standards, graded 0 to ++++, as defined below. In addition, particular attention was paid to the occurrence of hyperplastic nodules (*cf.* 11).

0 = No alveoli present.

\+ = Presence of isolated alveolar (and ductal) buds over the whole gland or somewhat denser alveolar growth in only part of a gland (Fig. 2).

++ = More extensive alveolar development and the presence of clusters of alveoli (small lobules) (Fig. 3).

+++ = Very heavy alveolar development with many small lobules or some large lobules (Fig. 4).

++++ = Extensive alveolar growth with many large lobules (Fig. 5).

(2) The mounted third pair of mammary glands from each mouse was projected on a screen at approximately 4× magnification, and a tracing of the perimeter of each gland was made. Using a planimeter, the areas in cm^2. were determined. The mean value and the standard error of the mean were computed for each group. The data presented graphically (see Fig. 1) demonstrate the extent of variation occurring within each group. The small size of the individual groups precludes further statistical analysis. Animals whose third pair of glands was torn or congenitally absent are not included in the graphs; hence, the number of mice indicated per group in Figure 1 is occasionally less than the total number of animals sacrificed, as shown in Table 1. Measurement by this method shows the change in gland area in each time period, and is used as an indication of extent of development of the duct system. It is recognized, however, that this method does not take into account the growth and branching of the ducts which occurs inside the perimeter of the gland.

RESULTS

The patterns of ductal and alveolar response to hormonal stimulation in the various strains and their hybrids may be summarized as follows (see also Table 1 and Fig. 1). The gland areas recorded for any single group varied greatly (Fig. 1). For this reason, only limited analysis can be made of growth relationships. On the other hand, alveolar responses within a group, and within the groups of any one strain, are fairly consistent throughout the experiment. This consistency despite the small numbers of animals in each group permits us to consider as significant the differences seen in the alveolar responses to the hormonal treatment.

In general, the glands of all experimental groups increase in size until about day 55, after which the fluctuations in size appear to be random.

No alveoli were present on any glands from control mice which did not receive injections of hormones.

C57Bl and C3H and their hybrids

In general, the ductal and alveolar responses of the hybrids show the same relationship to each other as do the respective responses of the two maternal strains. The untreated C3H gland at day 0 is smaller than that of the C57Bl, and the gland of the C3H×C57Bl hybrid is smaller than that of the C57Bl×C3H hybrid (see Figs. 8–11). Under hormonal stimulation,

the absolute increase in size of the C57Bl gland by day 15 is greater than that of the C3H (see Figs. 12 and 13). However, considering the more extensive degree of duct development in the untreated C57Bl gland, the C57Bl gland shows only a 2.4-fold increase in size by day 15, whereas the C3H gland increases to 8.4 times the size of its untreated control in the same time period.

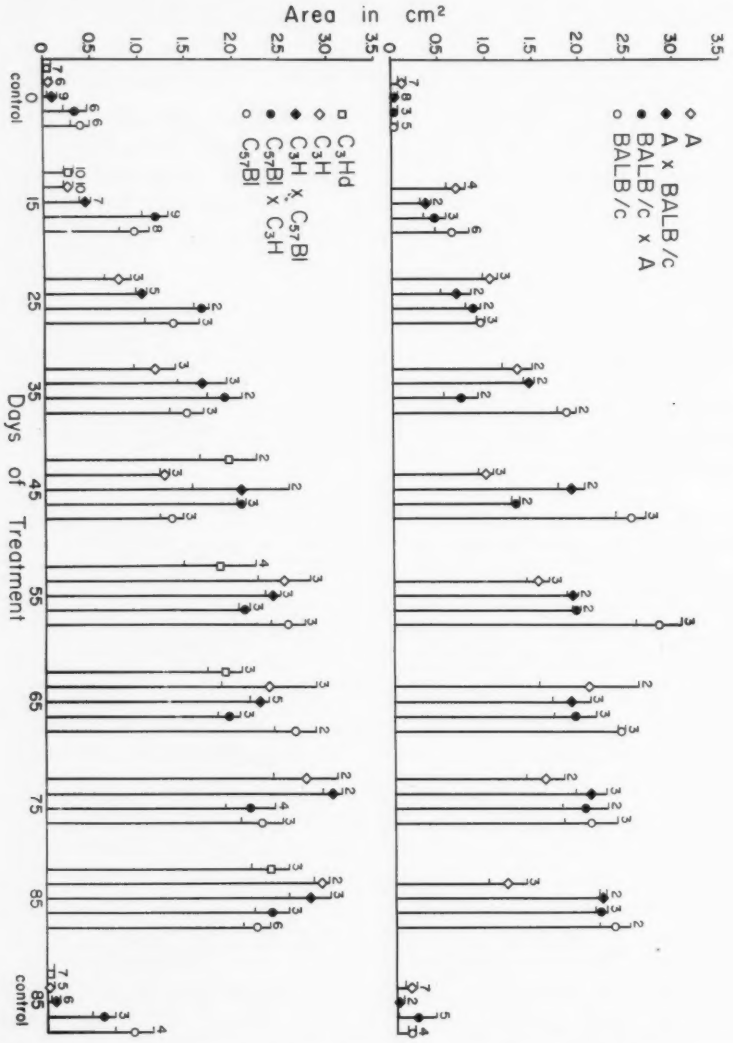


FIG. 1. Mean area of #3 mammary glands after stimulation with estrone and progesterone. Symbols on lines denote mean values for each group; cross-lines demarcate S. $E_{.m}$; numerals represent numbers of mice.

The alveoli of the C3H appear more rapidly than do those of the C57B1. At no time are the alveoli of the C57Bl as well developed as those of the C3H. Hyperplastic nodules were present in glands from all 3 of the C3H mice at day 85 (Figs. 6 and 7), at a time when there appear to be less alveoli than at day 75 and 65 despite continued hormonal treatment. The degree of alveolar development in the hybrids is less marked (except at day 85) than in the parent C3H strain, and this development occurs more slowly initially in the C57Bl×C3H hybrid than in the C3H×C57Bl hybrid, thus paralleling the relationship between their maternal parents. Two of the 4 C3H×C57Bl hybrids also show hyperplastic nodules at day 85, whereas the C57Bl×C3H hybrids show a striking increase in normal alveolar development at this time with no evidence of hyperplastic nodules.

C3H and C3Hd

Size differences between the C3H and C3Hd glands are not constant. At all stages the alveolar development is greater in the C3H than in the C3Hd. The hyperplastic nodules characterizing the C3H glands at day 85 are not found in the C3Hd.

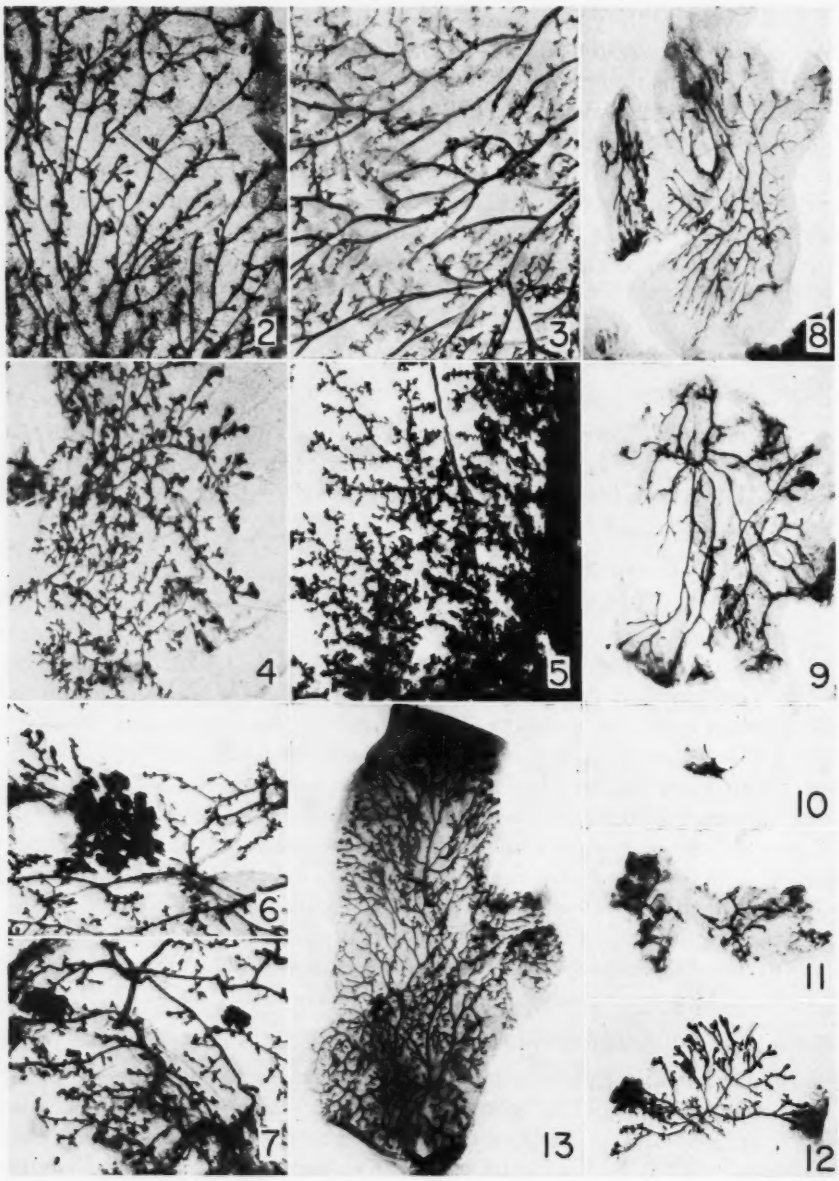
A and BALB/c and their hybrids

There is no change in the simple rudimentary duct system of the control glands of the A mice and of the A×BALB/c hybrid mice from day 0 to day 85; however, the BALB/c and the BALB/c×A hybrid control glands seem to undergo minimal ductal development during this time. Under hormonal stimulation both the absolute and the relative increase in size of the A gland is less than that of the BALB/c gland. Thus, at day 15, the glands are about equal in size, but the A gland is only 6 times its initial size, whereas the BALB/c gland is 18 times its smaller initial size.

Alveolar development is more rapid in the A than in the BALB/c until day 35, and it is also faster in the A×BALB/c hybrid than in the BALB/c ×A hybrid. By day 45 all four groups are nearly equal in alveolar development and remain so. The general pattern throughout the injection period is one of slightly less or slower alveolar development in the hybrids as compared to the parent strains.

General comparison of strains and hybrids

The four strains can be listed in order of ability to form alveoli under hormonal stimulation as follows: C3H > A > BALB/c > C57Bl. Because the untreated control gland of the C57Bl is larger than those of the other 3 strains, the C57Bl shows the least percentage increase in gland size by day 15, even though its glands at that time are still the largest of the strains. Early alveolar development in the hybrids tends to be less than that in any of the inbred strains studied except C57Bl; hybrids of the A and BALB/c show greater initial alveolar development than do hybrids of the C3H and C57Bl.



FIGS. 2–13. Whole mounts of mammary glands stained with alum-carmine. All mice, except the controls whose glands are illustrated in Figures 8–11, were treated with estrone and progesterone. Figures 2–7, ×7; figures 8–13, ×3.

DISCUSSION

Among the strains used, those with a genetic susceptibility to the development of mammary tumors, whether they possess the mammary tumor agent (A, C3H) or not (BALB/c), show more alveolar development than does the one strain used which lacks both these factors (C57Bl).

Considering all the strains and hybrids, no consistent correlation between the presence of the mammary tumor agent and the size of the mammary glands can be found, which we interpret as indicating little or no effect of the agent on ductal growth.

It is notable that in both hybrid series, the hybrid which received the mammary tumor agent from its maternal parent shows greater early alveolar development than its reciprocal lacking the agent, just as its maternal parent strain (possessing the agent) shows greater initial alveolar development than its paternal parent strain. Although there are other differences between the reciprocal hybrids, the role of the mammary tumor agent appears to be a real one, since elimination of the agent from the C3H (C3Hd) decreases the alveolar response markedly.

According to some authors, the mammary tumor agent influences both the mammary gland response to estrogen in castrate females (12) and the mammary gland morphology in the intact female mouse (13). Other workers (2, 14) claim that the mammary tumor agent does not influence the mammary gland response to estrogen. Among the castrate males treated with estrone and progesterone in the present study, hyperplastic nodules (*cf.* 11) were seen only in animals which have the mammary tumor agent (C3H and C3H×C57Bl). Our A strain, although it possesses the mammary tumor agent, does not ordinarily show nodules in the virgin female but only in parous animals (15), and nodules are not seen in the present study after estrone and progesterone treatment.

From Table 1 it appears that the alveolar development in some cases is greater at day 15 than at day 25 and even later. In these cases the apparent decrease in alveoli may result from duct growth in this period, and a consequent spreading of the alveoli present over a greater area. By day 85

FIG. 2. + alveolar development C57B1×C3H hybrid at day 65.
FIG. 3. ++ alveolar development. C57B1 at day 65.
FIG. 4. +++ alveolar development. C3Hd at day 65.
FIG. 5. ++++ alveolar development. C3H at day 65.
FIGS. 6 & 7. Hyperplastic nodules surrounded by ++ alveolar development. C3H at day 85.
FIG. 8. C57B1 control at day 0.
FIG. 9. C57B1×C3H hybrid control at day 0.
FIG. 10. C3H control at day 0.
FIG. 11. C3H×C57B1 hybrid control at day 0.
FIG. 12. C3H at day 15.
FIG. 13. C57B1 at day 15.

the glands are no longer enlarging (although terminal buds are still seen in some glands from hormone-treated animals of all strains at this time), and the reduction in alveoli in the C3H, in the C57Bl, and in the BALB/c probably represents a degree of involution despite continued treatment.

Treatment was not carried beyond day 85 because of the possible effect of sex steroids from the adrenal in the C3H strain, where nodular hyperplasia of the adrenal cortex is known to occur after castration (16, 17).

In general, then, the treatment of castrate male mice with ovarian steroids as used in this experiment results in alveolar responses of the mammary gland which show some relationship to the factors involved in mammary tumor development. Within the strains and hybrids used, those groups which possess genetic susceptibility to mammary tumor formation show greater alveolar response to the hormonal treatment. In addition, the alveolar response is further increased in the presence of the mammary tumor agent.

SUMMARY

Castrate male mice of strains C3H/HeCRGL, A/HeCRGL, C57Bl/CRGL, and BALB/c/CRGL, and of the reciprocal hybrids of C3H and C57Bl and of A and BALB/c, as well as mice of the C3H strain freed of the mammary tumor agent (C3Hd), were treated with 5 μg. estrone and 2 mg. progesterone on alternate days for periods up to 85 days, to produce appreciable lobulo-alveolar development. The mammary glands were evaluated with regard to general growth (based on area measurements of the third pair of glands) and to degree of alveolar proliferation. The one strain (C57Bl) which possesses neither genetic susceptibility to mammary tumor formation nor the mammary tumor agent shows the least alveolar response to the hormonal combination used. Within these strains and hybrids, the mammary tumor agent appears to influence the extent of alveolar development under the hormonal treatment, but shows little relation to overall gland growth. After 85 days of treatment hyperplastic nodules are seen in mice of the C3H strain and of the C3H×C57Bl hybrid.

Acknowledgments

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PLASMA KETOSTEROID STUDIES IN NORMAL MICE[1]

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ZIMMERMANN (1), Dumazert and Valensi (2), and Gardner (3) described methods for the measurement of neutral 17-ketosteroids (17-Ks) in human plasma. Migeon and Plager (4) described a method for the fractionation and measurement of 17-Ks in human plasma This report concerns the measurement of 17-Ks in mouse plasma.

The procedure was developed to facilitate the study of the relationship between plasma 17-Ks and tumor growth in transplantable mouse tumors. The method to be described has been applied to normal male and female Swiss Webster mice, adrenalectomized male and female Swiss Webster mice, ACTH stimulated Swiss Webster mice, normal female DBA_2 mice, and normal female C-57 black mice.

MATERIALS

1. 2N. alcoholic KOH prepared as described by Wilson (5).
2. Meta-dinitrobenzene recrystallized from alcohol. A 0.2% solution is prepared in redistilled ethanol.
3. Dehydro*epi*androsterone standards, 100, 200, 400 μg./ml. ethanol.
4. Purified corticotrophin gel, 40 U.S.P. units/ml. The National Drug Company, Philadelphia, Pennsylvania.
5. Constriction Pipettes were obtained from Arthur Thomas Co., Philadelphia, Pa.
6. Micro Cuvettes and diaphragm attachment for Beckman Spectrophotometer were obtained from the Pyrocell Manufacturing Company, New York City, New York.
7. Male and female Swiss Webster Mice were obtained from Blue Spruce Farm, Altamont, New York.
8. Female DBA_2 and female C-57 black mice were obtained through the courtesy of Dr. Michael Potter of the National Cancer Institute, National Institutes of Health.

HYDROLYSIS, EXTRACTION, AND COLOR DEVELOPMENT

The method of Gardner for hydrolysis and extraction of 17-Ks from human plasma (3) was modified for the study of mouse plasma. Three ml. of mouse plasma were pipetted into a 250 ml. Erlenmeyer flask to which 25 ml. of 10% aqueous HCl were added. This solution was boiled for ten minutes and transferred to a 250 ml. centrifuge bottle and extracted four times with 25 ml. aliquots of redistilled ether. The layers were separated by

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brief centrifugation. The combined ether extract was washed with three 25 ml. portions of 10% NaOH and the wash discarded. The ether was then washed with four 50 ml. portions of water. The ether was then taken to dryness on the steam bath. The residue was dissolved in 20 ml. of 70% ethanol and washed with three 15 ml. portions of petroleum ether. Ten ml. of distilled water were added to the ethanol layer and the solution extracted three times with 15 ml. portions of chloroform. The ethanol was discarded and the chloroform was reduced to approximately 10 ml. on the steam bath. This solution was then passed through a 10 mm. column consisting of 3 grams of Florosil previously washed with chloroform. The sample was followed with 30 ml. of chloroform. The combined effluent was evaporated to dryness on a steam bath and the residue taken up in 3 ml. alcohol and transferred to a 4 ml. test tube. Using a gentle stream of air, the alcohol was evaporated to dryness and the test tube walls washed down with 1 ml. alcohol which was evaporated to dryness.

COLOR DEVELOPMENT

For determination of the 17 Ks level in the plasma sample, a modified micro Zimmermann reaction adapted from that described by Wilson (6) was used. The color reaction was carried out on the samples, three standard dehydroepiandrosterone levels, and two column blanks. The column blanks were made by substituting pure chloroform for the chloroform solution of sample in the chromatographic procedure.

Reagents were added by means of constriction pipets to the tubes as indicated in Table 1. All tubes were chilled in crushed ice during the additions and exposure of the reaction mixtures to light was minimized. The tubes, still in a bath of crushed ice, were stoppered and placed in the refrigerator for exactly three hours. Then 0.2 ml. redistilled toluene was added to each tube with thorough mixing. A rare sample would show a brownish color and was discarded. Optical density was determined at 530 mu. in a Beckman DU Spectrophotometer against a blank containing toluene, absolute ethanol, and meta-dinitrobenzene in amounts proportional to that con-

TABLE 1. REAGENTS ADDED FOR COLOR DEVELOPMENT

	Dehydroepi-androsterone	Absolute alcohol	Meta-dinitro benzene	Alcoholic KOH
Column blank	0	20 μl.	20 μl.	40 μl.
2 μg. std.	20 μl. 100 μg./ml. DHA	0	20 μl.	40 μl.
4 μg. std.	20 μl. 200 μg./ml. DHA	0	20 μl.	40 μl.
8 μg. std.	20 μl. 400 μg./ml. DHA	0	20 μl.	40 μl.
Sample	0	20 μl.	20 μl.	40 μl.

tained in a plasma sample. Corrected optical densities of the plasma sample were derived by subtracting average optical densities of the column blanks from the optical densities of the individual plasma samples. Figure 1 shows the optical densities obtained from dehydroepiandrosterone by this pro-

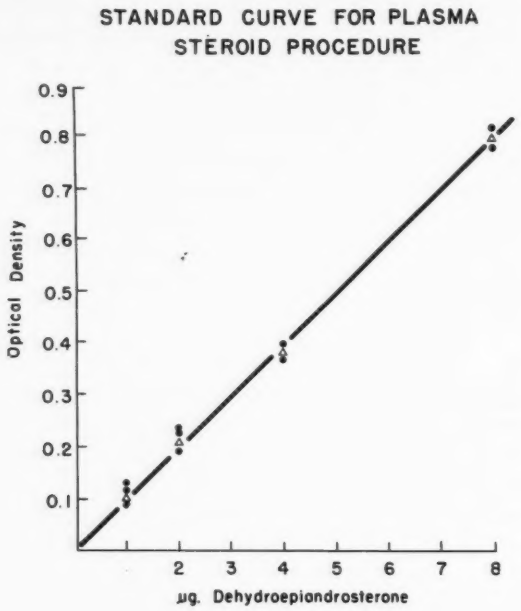

FIG. 1

cedure. Dehydroepiandrosterone added to plasma was recovered quantitatively within ten per cent.

MOUSE STUDIES

It was not possible to do a determination upon the plasma obtained from a single mouse with the present procedure. Each determination presented below represents a three ml. plasma sample obtained by pooling the plasma from four mice.

Ether anaesthesia was used for blood collection. An abdominal incision exposed the femoral artery which was cut with sharp scissors. The blood was drawn into a syringe containing a drop of heparin.

All mice were maintained on Purina Dog chow and tap water with the exception of the adrenalectomized animals which were maintained on Purina Dog chow and normal saline.

STUDIES ON SWISS WEBSTER MICE

Normal Swiss Webster male and female mice were bled at the age of two months. The plasma 17 Ks-level ranged from 0.50 µg./ml. to 0.61 µg./ml.

in the male samples and from 0.24 μg./ml. to 0.39 μg./ml. in the female samples. Swiss Webster male mice were adrenalectomized at the age of two months and were bled on the sixth postoperative day. The plasma 17-Ks levels ranged from 0.25 μg./ml. to 0.37 μg./ml., whereas determinations on adrenalectomized Swiss Webster female mice of the same age gave values from 0.00 μg./ml. to 0.03 μg./ml. Two month old Swiss Webster male mice were given one international unit of ACTH subcutaneously per day for a period of four days. These mice were bled on the fifth day after beginning

TABLE 2. PLASMA 17-KETOSTEROIDS IN NORMAL, ADRENALECTOMIZED, AND ACTH-STIMULATED MICE

Group	Strain	Sex	Number of pools[1]	Plasma 17-Ks μg. ml. ±S.D.
Intact	Swiss Webster	Male	7	0.550 ±0.043
Intact	Swiss Webster	Female	6	0.310 ±0.058
Intact	DBA_2	Female	5	0.360 ±0.027
Intact	C-57 Black	Female	5	0.340 ±0.045
Adrenalectomized	Swiss Webster	Male[1]	3	0.320 ±0.060
Adrenalectomized	Swiss Webster	Female	6	0.006 ±0.007
A.C.T.H.-Stimulated[2]	Swiss Webster	Male	5	0.850 ±0.050

[1] Each pool represents four mice sacrificed at two months of age.
[2] Injected subcutaneously with 1 I.U. ACTH daily for four days. Analysis on fifth day.

the injections and determination on these plasma samples revealed 17-Ks levels of 0.77 μg./ml. to 0.89 μg./ml.

Table 2 illustrates the analyses of normal, adrenalectomized, and ACTH-stimulated mice.

DISCUSSION

The data illustrate that 17-Ks can be measured in mouse plasma. It can be seen from the low values obtained from adrenalectomized female mice that little non-specific color is estimated by this procedure.

The development of color with m-dinitrobenzene at low temperature was adopted after trial of several simpler procedures. With color development at room temperature, many plasma samples gave brown colors which required determination of optical density at three wave lengths for satisfactory analysis. The clean purple colors obtained by the present procedure should make the determination of plasma 17-Ks on a sample from a single mouse possible by utilizing capillary cuvettes of 5 cm. light path. Such a procedure was described for plasma corticoid in the rat (7). The hydrolysis might be improved by substituting the more elaborate continuous ether extraction below pH 1 used by Migeon (4). The chemical nature of mouse plasma 17-Ks is under investigation.

SUMMARY

A method has been described for the measurement of 17-Ketosteroids in mouse plasma. Data are presented demonstrating plasma 17-Ketosteroid

values in intact, and adrenalectomized mice. The administration of ACTH increased the plasma 17-Ketosteroids as measured by this procedure. Adrenalectomized female mice have no significant plasma 17-Ketosteroid levels.

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THE COMPARATIVE ANTILUTEINIZING POTENCY OF PROGESTERONE DERIVATIVES WITH OXIDATION AT C-11, C-21 AND C-17

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THE antiluteinizing potency of progesterone (P) is diminished by those oxidations which occur during steroidogenesis in the suprarenal cortex. However, desoxycorticosterone (DOC), 11β-hydroxyprogesterone (11β-OH-P) and 11-keto-progesterone (11-keto-P) still possess antiluteinizing potency though in a lesser degree than progesterone (11, 14, 15). More recently we have studied the significance of the third site of oxidation characteristic of suprarenal steroidogenesis, i.e., 17α-OH.

METHODS

Experimental methods and classification of results have been the same as in the last of the papers quoted (15). In adult castrated females, weighing about 300 gm, one of the ovaries was grafted into the spleen. Two weeks later a pellet of estradiol was implanted beneath the skin. A pellet of the steroid whose antiluteinizing potency was under study, was implanted simultaneously. In former work animals were necropsied *six* weeks later; more recently necropsy has been performed already at two weeks after implantation.

RESULTS[1]

Results are expressed as a "*coefficient of luteinization*" which is the product of the average number of corpora lutea x and the average of the largest diameters of the corpora lutea y in the group. The coefficient $x \cdot y$ for 30 to 60 μg./day of estradiol is about 2.3 after six weeks, and about 1.7 after two weeks (column 0 in Fig. 1). When 23 or 39 μg./day of P are absorbed simultaneously with the estrogen during two weeks, corpora lutea are absent in most or all of the animals; $x \cdot y$ decreases to 0.2 or 0 respectively (the second zero point of the P groups in Fig. 1 refers to an experiment of six weeks' duration with 40 μg./day). DOC, or 21-OH-P, produces an antiluteinizing effect with 200 to 400 μg./day. With 47 μg./day of 11β-OH-P the result is similar to that obtained with 40 μg./day of P.

A seemingly complete loss of antiluteinizing potency of P is effected by oxidation of P at C-17: no antiluteinizing action was obtained with as much as 277 μg./day of 17α-OH-P, i.e. 7 to 10 times the antiluteinizing quantity of P (12). There was no antiluteinizing action with similar quantities of the caproic ester of 17α-OH-P (213 μg./day).

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[1] All figures referring to μg./day are the averages of the groups.

None of the three 17α-OH cortical compounds—Reichstein's cpd.S, cortisone (E) or hydrocortisone (F)—had any antiluteinizing action. Large quantities of S acet. (427 μg./day), E acet. (1742 μg./day), or F acet. (1600 μg./day; free 5140 μg./day), 12, 50 or 100 times those of P, were still ineffective (Fig. 1).

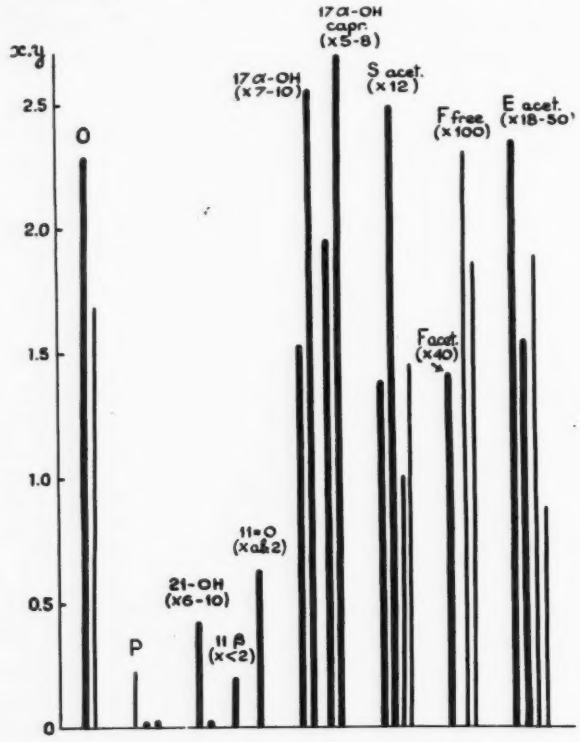

FIG. 1. *Suppression of the antiluteinizing potency by oxidation* 17α-OH. Height of columns: coefficient of luteinization. Each column corresponds to a group of 3 to 15 animals. There were 24 groups totalling 147 animals of which 57 are taken for comparison from former papers. Thick lines ━ experiments of six weeks duration; thin lines — experiments of two weeks duration. *All* animals received 30–60 μg./day of estradiol. Column 0—animals receiving estradiol but no progesterone or progesterone derivative. Figures in brackets on the top of columns: quantity of the respective steroid expressed in approximate multiples of the quantity of progesterone sufficient to prevent estrogen-induced luteinization.

DISCUSSION

When progesterone is transformed in the suprarenal cortex into corticoids through oxidation at C-21, C-11 and C-17, the various activities inherent to P break down. From the point of view of two antiestrogenic potencies—antifibromatogenic, antihysterotrophic—oxidation at C-17 has

been shown in former work to be crucial, both these potencies being abolished (12, 16). The results of the present study indicate that the same is true also for another antiestrogenic potency—the antiluteinizing one. It would seem that it is the same with the comparative progestational potency of the oxy-derivatives of P. DOC still retains about one tenth, and 11β-OH-P about one thirtieth to one tenth of the progestational potency of P (1), and both are still antiluteinizing. On the contrary no progestational action has been obtained with 17α-OH-P (7, 17); this compound, and likewise its hydroxy-derivatives (cpd.S, hydrocortisone), are devoid of antiluteinizing potency as shown above.

In view of the outstanding work of the Worcester group on steroidogenesis in the suprarenal cortex (9, 18), the above statements about the functional changes effected by oxidation of P may be generalized in the following way: the crucial functional change from luteoid to corticoid is effected by oxidation at C-17—the various potencies of P all break down, but corticoid functions are acquired only by previous or successive oxidation at C-21 and C-11.

These general conclusions are based on work with the comparative antiestrogenic potencies of pregnene derivatives in the *guinea-pig*. Experiments in *rats* gave contradictory results. In hypophysectomized rats receiving estrogen an antihysterotrophic action was obtained with 9α-f-11β, 17α-di-OH-P and with 9α-f-hydrocortisone (6); results were similar even with non-halogenated hydrocortisone (21). Various authorities found that progesterone facilitates ovulation and corpus luteum formation (3, 4, 8; summary 2). With quantities as large as 2000 μg./day there was a prolongation of the estrogen-induced vaginal cornification (13).

On the other hand in more recent work (19) there was, in rats and rabbits, a definite antiovulatory action of progesterone, 19-norprogesterone, ethinyltestosterone and 19-nor-ethinyltestosterone, and of various other steroids. It is remarkable that, in the rat, also 17α-hydroxyprogesterone was active in suppressing ovulation.

It is known that luteinization fails to appear in ovaries grafted into castrated male guinea-pigs (intrarenal grafts; 10) or rats (intraocular grafts; 5). However luteinization takes place when, in castrated rats with an ovarian graft, suprarenalectomy is performed (20). The results of the present study suggest that suprarenalectomy in castrated animals removes an antiluteinizing factor.

SUMMARY

Progesterone derivatives with oxidation at C-21 (DOC) and C-11 (11β-hydroxyprogesterone, 11-ketoprogesterone) retain, in guinea-pigs, a certain amount of antiluteinizing potency. No such potency is retained by derivatives with oxidation at C-17 (17α-hydroxyprogesterone. Reichstein's compound S, hydrocortisone, cortisone). Since 17α-hydroxypro-

gesterone is, in the guinea-pig, devoid also of other antiestrogenic potencies and, in the rabbit, devoid of progestational potency, oxidation of progesterone at C-17α can be considered as a definite step on the way from luteoid to corticoid functions.

Acknowledgments

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THYROID AND OVARIAN FUNCTION IN RELATION TO MOLTING IN THE HEN

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IT IS well known that the administration of thyroid substance induces molting in the hen in any season of the year (1, 2, 3). Some investigators reported that histological examination revealed an activation of thyroidal function during the natural molting period in several species of birds (4, 5) or in pre-molting period (6). These findings have naturally led to the supposition that molting is initiated by an increased activity of the thyroid gland.

Sulman and Perek (7) showed, however, that thiouracil feeding precipitated molting in the hen. Shaffner (8, 9) reported that the administration of a large amount of progesterone induced unseasonable molt not via the thyroid in the hen. Furthermore, Tanabe *et al.* (10) reported no appreciable increase in the uptake of radioiodine by the thyroid was observed in molting hen compared with laying hen. These results throw much doubt on the dependence of molt on the thyroid.

With these seemingly contradictory facts in the background, to clarify the mechanism of molt, we measured the rate of release of thyroid hormone from the thyroid both in molting and laying periods of the hen. At the same time the ovarian function was also noted.

MATERIALS AND METHODS

Ten yearling Single Comb White Leghorn hens weighing 1.5–1.8 kg. were used. Each hen was placed in a single metabolism cage in a temperature-controlled room at 16–20° C (average 18° C), for the period from September 15 or 25 to December 16, under natural daylight. The hens were fed *ad libitum* on the standard diet of the Institute. One hen was starved for 5 days, but was allowed to take water *ad libitum*. The hens were injected intravenously with 8–20 μc. of carrier free inorganic I^{131} [1] in 0.3–1.0 cc. isotonic saline. Forty mg. of crystalline progesterone[2] dissolved in olive oil was injected once or twice intramuscularly. TSH (Pretiron)[3] was dissolved in 0.9% NaCl solution and immediately injected subcutaneously. The dose was 12.5–50 Junkmann-Schoeller U. (20–80 mg. U.S.P. eq.).

Measurement of thyroid activity by the rate of release of I^{131} was performed by a method similar to those used by Wolff (11) and Albert (12) with the rat and by Brown-Grant (13) with the rabbit. The unanaesthetized hens were placed in the immobilization apparatus during the measurement of radioactivity. The γ-radiation from the thyroid

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[1] Carrier-free I^{131} was furnished from the Radiochemical Center, Amersham, England.

[2] Progesterone was supplied by Biorganic Laboratory Inc., U.S.A.

[3] TSH (Pretiron) was supplied by Schering A.G., Berlin.

region was counted with a scintillation counter[4] with a round opening (diameter 40 mm.) in the hollow cylindrical lead shield (thickness 50 mm., length 250 mm.), under constant geometrical conditions, the distance from the surface of the crystal in the counter to the thyroid region of the hen being 150 mm. Background counts were about 80 per minute. The hens were injected with I^{131} repeatedly when the counting rate fell to less than 4 times the background, the usual counting range being 500–10,000 counts per minute. The mean variation between repeated counts was 2.5% of the total counts. Counts over thyroidal area in vivo started 48 hrs. after the injection of I^{131}, observations being made once daily for periods of 9–30 days. The counts corrected both for background and for physical decay were plotted semi-logarithmically versus time. When plotted semi-logarithmically the points fell on a straight line, the slope of which represents the ratio of the amount of hormone secreted per unit time to the total amount in the gland. Therefore provided that the amount of hormone in the gland remains constant, changes in the slope will be related to changes in thyroid activity. In preliminary tests some birds were killed on 4th, 11th and 22nd days after the injection of I^{131}. The thyroids were removed immediately, and the athyreotic carcasses were counted under identical conditions. In each case the radioactivity was negligible in the carcasses.

Ovarian activity of the hens was measured by the immunological method by Hosoda *et al.* (14), examining the presence of serum vitellin. The serum vitellin content was expressed in terms of the logarithm of the highest dilution of the serum of the hen giving a positive reaction. Individual egg production was recorded during the experimental period.

In order to examine the molting process of the hens, the number of the feathers cast off on the floor of the cages and the primary wing feathers lost per wing were counted

RESULTS

The release rate of I^{131} from the thyroid, the record of egg production, the log titer of vitellin reaction representing ovarian activity, and molting process in each bird for the period of September 29–December 16 are illustrated in Figures 1–3. At the start of the experiments (September 27) all the hens were laying. Molting began in five hens out of six in a period from October 13–November 8. One hen which did not molt by early December was forced to molt by progesterone (Fig. 1). In each bird molt, including forced-molt, occurred on 4th-8th day after the cessation of lay. Whenever the hen resumed egg production even in the course of molting the shedding of feathers stopped immediately and completely.

Any acceleration in the output of I^{131} from the thyroid was not observed either in molting or in pre-molting period (Figs. 2, 3). The slope of the release curves varied considerably with different hens. Expressed as percentage loss of thyroidal radio-iodine per day the variation was 2–8% (average 3.5%). The value is very low, compared with 17% in rabbit (13), and 10–20% in rats (11, 12).

The injection of TSH increased the rate of release by seven times, the percentage loss of thyroidal I^{131} being 15% per day (Fig. 2). This result proves that the method based on the release of I^{131} from the thyroid is a reliable technique for the study of thyroid activity in the fowl. Starvation

[4] Kagaku-Kenkyujo (Scientific Research Institute) Ltd., Tokyo.

completely inhibited thyroidal I^{131} release (Fig. 3). Two injections of 40 mg. of progesterone induced molt in the hen, but somewhat decreased the release of thyroidal I^{131} from the thyroid. Serum vitellin titer diminished and approached to zero whenever lay ceased.

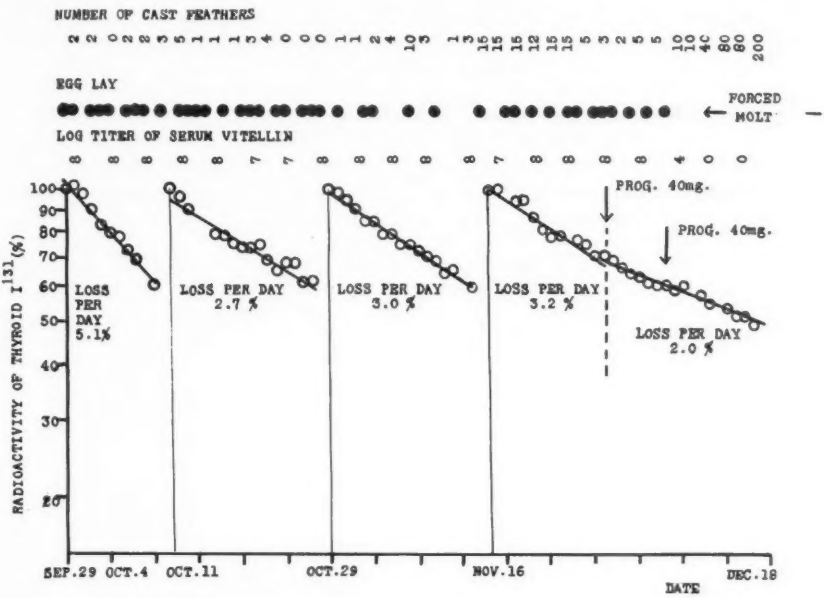

FIG. 1. The secretion rate of thyroid hormone measured by the release rate of I^{13} from the thyroid, egg production, molting process expressed by number of cast feathers and ovarian activity expressed as serum vitellin content of hen (No. 186).

In most of the birds in the 2–3 week period following the first injection of I^{131}, the rate of loss of thyroidal I^{131} was rapid, but in the repeated experiments the rate was not so rapid as in the first period. It seems that the increased thyroid activity observed in the first period of the experiment was not related to the molt which occurred 1–2 months later. Since the activation in the first 2-week period was also observed in the hen which ultimately did not molt (Fig. 1). Furthermore, the time interval from the start of treatment by thyroid hormone and progesterone to the initiation of molt in the hen is 1–2 weeks (3, 8, 15). These results diminish the possibility of the presence of an increased activity of the thyroid gland in the pre-molt interval.

DISCUSSION

The present study demonstrated that no increase in the release rate of thyroid hormone occurred in either the pre-molting or molting period compared with the laying period. Furthermore we have demonstrated

that no difference in thyroidal I^{131} uptake exists between the molting hens and laying hens (10). Sulman and Perek (7) and Himeno and Tanabe (15) showed that the laying hen stopped egg production soon after taking 0.5–1.0% thiouracil in food and began to molt 2–4 months earlier than untreated control. These results indicate that there is no relation between the inception of molting and the change in thyroid activity at least in the hen.

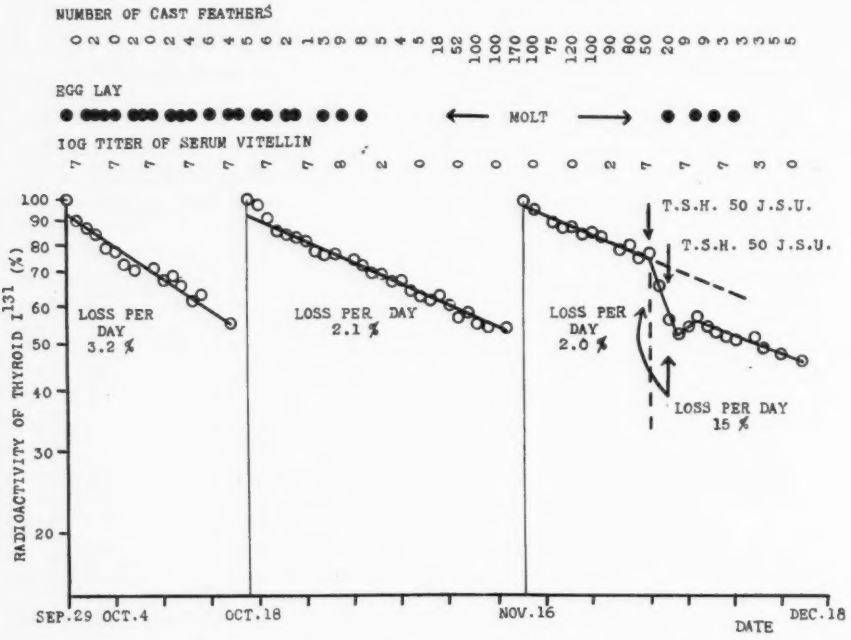

FIG. 2. The secretion rate of thyroid hormone measured by the release rate of I^{131} from the thyroid, egg production, molting process expressed by number of cast feathers and ovarian activity expressed as serum vitellin content of hen (No. 193).

The present study has demonstrated that molt never occurs while the hen is laying. Onishi *et al.* (16) observed that daily injection of 2.5 mg. stilbestrol completely prevented the progress of molt in the hen. Furthermore, it has been already observed that the treatments by thyroxine (3, 15), progesterone (9, 15), thiouracil (7, 15), Enheptin (2-amino,5-nitrothiazole:17,18) and starvation (19), all of which induce molt in the hen, suppress ovarian activity. These results suggest that the primary cause of natural molt in the hen may be a decrease in ovarian activity.

It was noted that natural molt is difficult to induce in early spring in the hen which had completed the preceding autumnal molt even if they stopped laying. Thyroxine (18) and progesterone (15) can induce molt in

such a hen, while thiouracil (15), Enheptin (18) and starvation (18) have no such effect, and it was found in the present study that progesterone had a slightly depressing effect on thyroid function. It seems, therefore, that the injection of high doses of thyroxine and progesterone can induce molt possibly by a direct activation of feather follicle.

However, it is highly probable from the results of the present study that natural molt in the hen is not induced by the increased release of thyroid

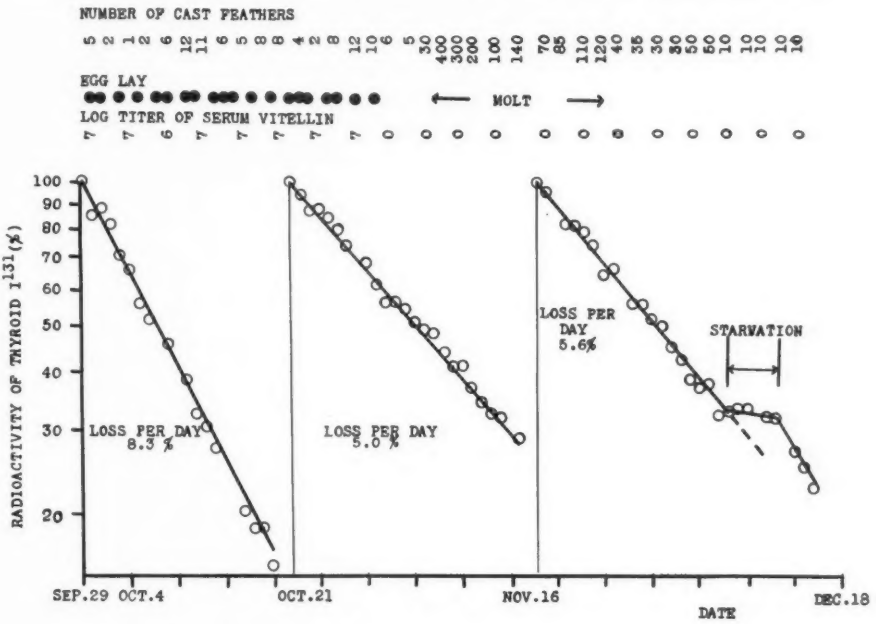
NUMBER OF CAST FEATHERS
5 2 1 2 6 12 11 6 5 3 3 4 2 3 12 10 6 5 30 400 300 200 180 140 70 85 110 120 40 35 30 50 50 10 10 10 10
EGG LAY
MOLT
LOG TITER OF SERUM VITELLIN
7 7 6 7 7 7 7 7 0 0 0 0 0 0 0 0 0 0 0
RADIOACTIVITY OF THYROID I^{131}(%)
100
90
80
70
60
50
40
30
20
LOSS PER DAY 8.3 %
LOSS PER DAY 5.0 %
LOSS PER DAY 5.6%
STARVATION
SEP.29 OCT.4
OCT.21
NOV.16
DEC.18
DATE

FIG. 3. The secretion rate of thyroid hormone measured by the release rate of I^{131} from the thyroid, egg production, molting process expressed by number of cast feathers and ovarian activity expressed as serum vitellin content of hen (No. 194).

hormone into blood stream, and possibly not by that of progesterone because the dose required to induce molt is unphysiologically large (9, 15, 20). These facts suggest a hypothesis that natural autumnal molt in the hen is induced by a decrease in the ovarian activity, but is not induced by the increased activity of the thyroid gland of the hen.

SUMMARY

The secretion rate of thyroid hormone in the hen during laying and molting periods was measured by the release rate of I^{131} from the thyroid. Ovarian activity of the hen was also determined by the immunological method.

The secretion rate of thyroid hormone was low. The average value ex-

pressed as percentage loss of thyroidal radioiodine per day was 3.5%. Acceleration in release rate of I^{131} from the thyroid was not observed either in molting or in pre-molting period. Molt occurred, whenever lay ceased and ovarian activity decreased.

TSH administration produced a marked increase in release of I^{131}. Progesterone administration forced the hen to molt, but somewhat decreased the release of I^{131}. Starvation completely inhibited the release of I^{131}.

It is suggested that natural autumnal molt in the hen is induced by decrease in ovarian activity, but is not induced by the increased activity of the thyroid gland of the hen.

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ATTEMPTS TO ALTER THE CONCENTRATION OF MELANOPHORE HORMONE IN THE PITUITARY GLAND OF THE RAT[1]

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RECENT literature contains several reports concerning the mechanism involved in the endocrine control of melanogenesis in man (1–5). All recent observers emphasize a relationship between adrenal cortical steroids and the melanophore hormone (MH, intermedin, and other terms) of the pituitary gland. It is postulated that the adrenal cortical steroids exert an inhibiting influence on the release or formation of MH. In conditions with decreased or absent adrenal cortical function, excessive amounts of MH are presumably formed and released into the blood stream, resulting in excessive melanogenesis. This concept is supported by the observation that MH is present in the blood of patients with a variety of clinical disorders characterized by intensive melanosis. However, the significance of these observations, since they are based on bio-assay of blood or urinary MH, is not known with certainty.

Animal experimentation has not always revealed results consistent with the foregoing concept of MH control by adrenal cortical hormones. Forgács (6) assayed the pituitary glands of rats treated with a cortisone preparation and found that, while the corticotropin content of the glands was decreased, the MH content was unaffected. Sulman and Evitar (7), studying the pituitary glands of dogs treated with cortisone, found marked suppression of MH content in the anterior lobe but no suppression of MH content in the middle and posterior lobes. This led them to postulate the existence of at least two MH hormones or factors, which were under separate controlling mechanisms. Other aspects of the possible regulatory mechanisms for MH secretion were studied by Karkun and associates (8), and by Frieden (9). The former treated kittens with corticotropin and observed histologic evidence of stimulation of the basophils of the intermediate lobe, while the latter reported a decrease in the pituitary content of MH following injection of MH into rats.

Hormones other than those from the adrenal cortex and pituitary gland affect MH or melanogenesis. To cite only a few examples, Frieden (9)

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demonstrated a decreased MH content of the rat pituitary gland following thyroidectomy, and Trinkaus (10) showed that thyroid hormone was necessary for normal pigmentation of the feathers of the brown Leghorn chicken. Koneff and associates (11) demonstrated marked hypertrophy of the intermediate lobe of hamsters after prolonged administration of diethylstilbestrol. Androgens are necessary for a normal response to ultraviolet irradiation in man (12) and also cause melanosis when applied locally to the scrotum of the ground squirrel (13) or to the skin-bill margin of the sparrow (14). Local application of estrogen to the nipples and areolae of castrated male guinea pigs leads to increased melanin formation, as does parenteral administration of estrogen (15).

The present study was undertaken in an attempt to determine whether or not the content of MH in the pituitary of the rat could be affected by various alterations in the endocrine status of the animal. Some of our results are in agreement with recent reports (6, 7), which appeared after this work was completed.

MATERIAL AND METHODS

Bio-assay of MH.—The following method of bio-assay for MH was used in this study. Strips of skin were removed from the legs of freshly killed frogs (Rana pipiens) and cut into small rectangular pieces. Each frog yielded approximately 16 such pieces. The skin was then thoroughly rinsed five times in frog Ringer's solution and allowed to remain in fresh Ringer's solution for an hour before being used. During this period the melanophores assumed a fully "contracted" state. Aliquots of the material to be assayed were placed in Petri dishes, and frog Ringer's solution was added to make a total volume of 20 cc. Ten pieces of frog skin, taken at random, were then placed in each Petri dish and the entire contents were mixed thoroughly. After 90 minutes, the dish was placed on the stage of a dissecting microscope and the response of the melanophores was observed. Rather than grading the melanophore "expansion" in stepwise fashion (16), any piece of skin which showed a definite response (dispersion of pigment) was graded as positive, and those pieces whose melanophores showed no change were judged negative. A quantal type of assay was, therefore, employed.

Lacking an International Standard preparation, the values obtained were expressed in terms of a unit which was arbitrarily adopted because of its suitability and convenience for this study. One unit of MH, as used herein, refers to that amount of MH which, when diluted to 20 cc., will cause a detectable melanophore response in five of 10 pieces of isolated frog skin. In effect, the dose of MH corresponding to the ED_{50} was regarded as one unit.

Using a stable preparation of corticotropin (Armour, J 19907 R) which was heavily contaminated with MH, dose-response curves were established. Three to four doses of this preparation were tested simultaneously, and the percentage of pieces of skin in each dish showing a positive response was plotted on semilogarithmic paper. A straight line was then drawn which visually best fitted all the points. This was done separately 35 times and the angle which this line made with the abscissa was measured. These angles varied from 39° to 66°; the average angle was 55°. It will be noted from Figure 1 that, although the slopes of the lines are all roughly the same, the position of the curve varies.

Male Sprague-Dawley rats were used exclusively. They were killed by a sharp blow on the head followed by severing of the spinal cord. The pituitary gland was quickly

removed, weighed and then homogenized in a small test-tube homogenizer. The pituitary homogenate was removed with at least 20 successive rinsings with hot tap water (temperature 57–59° C) and diluted appropriately for assay. The dilution was estimated on the basis of the pituitary weight. The stability of this preparation is not known, but it was always used for assay within 30 minutes. The MH content of rats treated with various hormone preparations was compared with that of normal animals maintained as controls. Four doses of the control pituitary were simultaneously tested, as described,

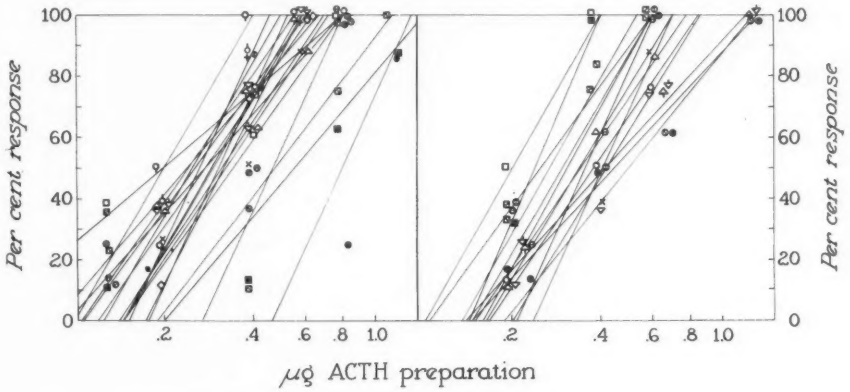

FIG. 1. Log-dose response relationship for 35 individual assays on a preparation of corticotropin (Armour, J 19907 R). Right side of figure illustrates 15 assays and left side illustrates 20 assays.

with four doses of the pituitary of the experimental animal. The points were fitted visually by a line subtending a 55° angle, and ED_{50} was read off. The ED_{50} values were converted into total MH units per pituitary and into MH units per milligram of pituitary tissue by the appropriate dilution factor and pituitary weight. Thus, an eight-point assay was carried out.

The effect of cortisone (cortone, Merck), hydrocortisone (hydrocortone acetate, saline suspension, Merck; solu-cortef, water-soluble, Upjohn), desoxycorticosterone acetate (percorten, Ciba), MH preparation (Armour, Lot No. R527109), adrenocorticotropin (H. P. ACTHAR Gel, Armour), thyroxine, and conjugated estrogens (premarin, Ayerst) was studied. Also, the effect of adrenalectomy was investigated, as was the effect of administration of corticotropin or hydrocortisone in adrenalectomized animals. Studies were undertaken to detect a diurnal variation in pituitary MH content and to determine the effect of blindness and environmental background.

All hormones were administered by subcutaneous injection. The rats in the control and in the experimental groups were of approximately the same size and age.

RESULTS

The results of injecting various hormones on the content of melanophore hormone in the pituitary are shown in the Table. Ten animals were treated with daily injections of 2.5 mg. of cortisone. Each day, one cortisone-treated animal and one control animal were killed and their pituitaries were assayed. It was noted (Fig. 2) that the MH values appeared to sep-

EXPERIMENTAL ALTERATION OF CONCENTRATION OF PITUITARY MH

Number of rats	Material	Daily dose, mg.	Pituitary MH concentration,* units/mg.	Body weight (at time of assay), gm.
10	Control	0	269±36.0	147± 7.0
10	Cortisone	2.5	262±45.4	102± 2.1
12	Control	0	234±25.5	181± 9.2
9	DCA	1.0	201±15.5	200±11.9
9	Hydrocortisone (suspension)	2.5	138±17.7	100± 4.1
10	Control	0	296±41.2	167± 4.4
10	Hydrocortisone (soluble)	2.5	164±20.8	167± 5.1
10	Control	0	240±20.5	172± 3.7
10	Hydrocortisone (soluble)	20.0	147±16.6	142± 2.6
10	Control	0	238± 8.4	167± 3.1
10	MH	2.0	207±12.5	167± 2.4
10	Control	0	253±33.9	181± 5.6
10	Corticotropin (gel)	2.0	156±27.8	164± 3.3
15	Control	0	220±23.9	163±12.0
8	Estrogen	2.0	229±43.2	111± 6.6
8	Thyroxine	0.1	191±27.1	157±14.7
10	Control	0	253±29.6	180± 4.3
10	Adrenalectomized	0	318±50.0	134± 7.4
10	Adrenalectomized plus corticotropin (gel)	2.0	371±42.9	124± 7.5
10	Control	0	224±32.2	187± 4.8
10	Adrenalectomized	0	254±33.7	140± 4.6
10	Adrenalectomized plus hydrocortisone (soluble)	2.5	402±38.6	145± 7.7
8	Night assay	0	267±27.3	—
3	Control	0	251±15.5	—
5	Optic nerve transection	0	240±19.9	—
5	White background	0	285±53.3	125± 4.5
5	Black background	0	295±41.0	130± 3.1

* Mean ± standard error.

arate from the sixth to eleventh days, two more or less distinct but overlapping groups appearing, with the cortisone-treated group having the lower concentration. The average for the 10 animals in each group was, however, not significantly different: 262 units/mg. for the cortisone-treated and 269 units/mg. for the controls.

Nine animals treated with daily injections of 2.5 mg. of hydrocortisone suspension had a mean pituitary MH concentration of 138 units/mg.; 12 control animals had a mean concentration of 234 units/mg. ($p = 0.01$). This decline of concentration became apparent after the eighth day of hormone treatment (Fig. 3).

Because the procedures described in the two preceding paragraphs indicated that changes in pituitary concentration of MH required approxi-

mately a week to occur, assays were not begun until the seventh day or later in all subsequent procedures.

A water-soluble hydrocortisone preparation was administered daily in doses of 2.5 mg. to 10 rats for 10 days, and the entire group of 10 treated rats and 10 controls was assayed over the next 3 days. The average values were 296 units/mg. for the 10 controls and 164 units/mg. for the 10 animals receiving the hormone ($P = 0.02$).

In an effort to decrease more effectively the concentration of MH in

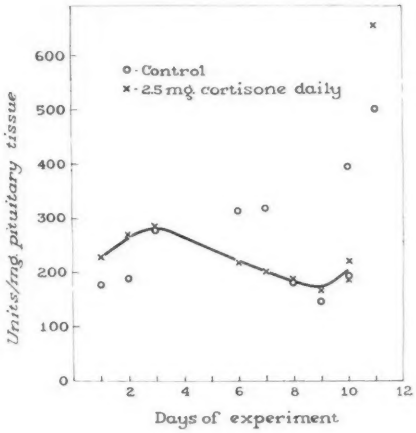

FIG. 2. Pituitary MH concentration in cortisone-treated and normal rats. Note generally lower values for cortisone-treated rats on and after the sixth day.

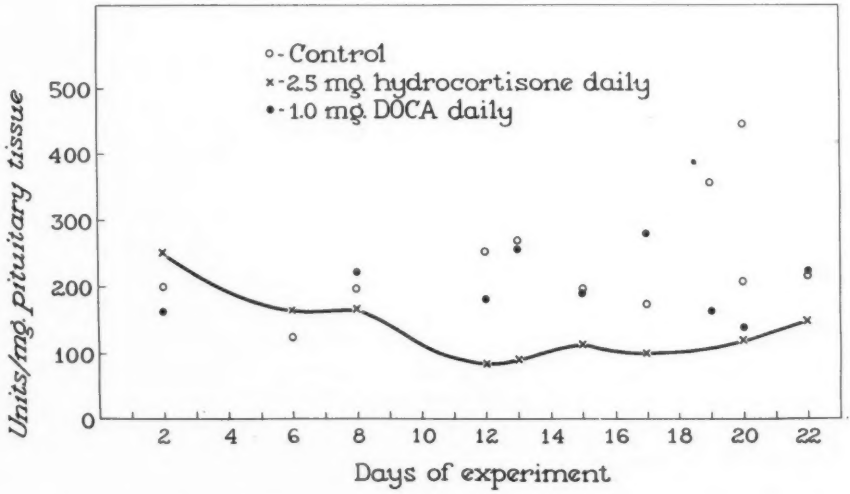

FIG. 3. Pituitary MH concentration in hydrocortisone-treated, DCA-treated, and control rats. Note generally lower values for hydrocortisone-treated rats after the eighth day as compared with values for DCA-treated and control animals.

the pituitary, massive doses of hydrocortisone were employed. Ten animals received 10 mg. of soluble hydrocortisone twice daily for 10 days. The values for the control and the treated groups were respectively 240 units/mg. and 147 units/mg. ($P < 0.01$).

Adrenocorticotropic hormone administered for 11 to 15 days to 10 intact rats in daily doses of 2.0 mg. led to a mean pituitary MH concentration of 156 units/mg.; the 10 control animals had a mean value of 253 units/mg. ($P = 0.05$).

Groups of rats were also treated with MH, thyroxine, estrogen, and desoxycorticosterone for periods ranging from 14 to 24 days. The mean pituitary MH concentration of the treated groups was almost identical with that of the control groups.

Ten bilaterally adrenalectomized rats demonstrated a mean value of 318 units/mg., 10 adrenalectomized animals who also received 2.0 mg. of corticotropin daily for 7 to 12 days had a mean pituitary MH concentration of 371 units/mg., and 10 control animals had 253 units/mg. The animals were operated on 9 to 14 days prior to assay. The P value for the difference between the controls and adrenalectomized animals was 0.3; the P value for the difference between the controls and the adrenalectomized animals which also received corticotropin was 0.05. A similar experiment was performed using hydrocortisone in place of corticotropin with similar results.

A diurnal variation of pituitary MH content was not observed when a small group of normal animals was assayed at night. The values compared well with control values obtained in the morning. Surgically produced blindness (transection of optic nerves 7 to 10 days before assay) and 6 to 7 days' exposure to constantly illuminated white and black backgrounds appeared to have no effect on pituitary MH content.

Nine groups of 10 or more control animals each were used throughout this entire study, comprising a total of 97 animals. The mean values of these nine groups ranged from 220 units/mg. to 296 units/mg. The mean of all these values was 246 units/mg., which probably represents the average normal value of MH concentration of the rat pituitary. The individual determinations which comprise these mean values fluctuate widely, however. Seventy-one per cent of the assays fell within 100 units more or less than the mean value, 246 units/mg. The remaining 29% of the readings ranged from as low as 104 units/mg. to as high as 515 units/mg. The values in the treated groups of rats were similarly distributed. No consistent relationship was observed between the pituitary concentration of MH and body weight, or pituitary weight, or the age of the animal.

COMMENT

The increased pigmentation of the skin and buccal mucosa of patients with primary adrenal insufficiency, and the decreased skin pigmentation

of patients with pituitary insufficiency have led several authors (17, 18, 19) to suggest that pituitary melanophore hormone has a controlling influence on human melanogenesis. Addisonian pigmentation following administration of pituitary preparations to man was first reported by Sprague and associates (20) in a patient with rheumatoid arthritis being treated with corticotropin. Because pigmentation occurred in the absence of adrenal insufficiency, it was logical to conclude that the cause of the pigmentation was some substance in the commercial corticotropin. Of the various contaminants of preparations of corticotropin as manufactured before 1950, the most likely contaminant that could conceivably induce melanogenesis was melanophore hormone. This likelihood was based on the close similarity of the then known chemical properties of melanophore hormone and of corticotropin. Indeed, assays carried out on hypophysectomized frogs showed the presence of large amounts of melanophore hormone in the preparation of corticotropin used in the foregoing clinical studies. Typical addisonian pigmentation has also been induced by melanophore hormone in a patient with generalized pituitary insufficiency without concomitant and marked hyperadrenocortisonism (Salassa and associates, 21). These and other observations (1, 2) indicated that melanophore hormone is capable of producing directly or indirectly intense melanogenesis in human skin. It was on this framework that evidence, based on bio-assays of MH in blood and urine of addisonian patients with and without cortisone therapy, was gathered for the concept that the adrenal glands control the production and secretion of melanophore hormone, thus regulating human melanogenesis.

The present work is pertinent to the adrenal regulation of melanophore hormone only to the extent that the changes reported for blood and urinary MH would eventually be reflected in similar changes in concentration of MH in the pituitary gland. With this limitation, our results do not lend support to the concept that an adrenal-MH axis is present or, if it is, that it operates with the same dispatch as the adrenal-corticotropin system. Granting somewhat generous limits for the inaccuracy of the MH assay as used herein (which, incidentally, is also true of assay methods in studies dealing with clinical material), our results show that certain adrenal steroids induce either no change in pituitary MH or, at best, a small reduction after prolonged exposure with massive doses. Whether these small changes, including the slight increase in MH after adrenalectomy, represent the contribution of corticotropin to total MH activity is not certain.

On the other hand, there is already excellent evidence (6) under generally similar experimental conditions that adrenal steroids produce the expected reduction in the corticotropin concentration of the pituitary without effecting a simultaneous depression of MH concentration. The reverse situation, that of adrenal insufficiency, is equally unfavorable to the existence of an adrenal-MH axis. The behavior of MH in the blood of

adrenalectomized rats is at the moment controversial, since Sulman (22) found no change, whereas Johnsson and Högberg (5) obtained a fourfold increase of blood MH. Our results show no significant increase of pituitary MH after adrenalectomy. In contrast to this negative or controversial effect of adrenalectomy on the behavior of MH, Sydnor and Sayers (23) have reported that both the blood and the pituitary content of corticotropin are elevated after adrenalectomy. Thus, it is somewhat difficult to accept as a general concept the presence of an adrenal-MH axis similar in operation to the adrenal-corticotropin axis.

SUMMARY

The administration of thyroxine, estrogen, MH and DCA to normal rats caused no major deviations in pituitary MH concentration from control values. Surgically blinded rats had normal values, as did those animals exposed to black or white backgrounds. No evidence was produced to indicate a diurnal variation in pituitary MH content.

Hydrocortisone and corticotropin administered to the intact rat produced approximately a 40 per cent reduction of the pituitary MH concentration. This depression due to hydrocortisone occurred after 7 days of treatment and appeared to be a maximal effect within the time limits of the experiment.

Adrenalectomy caused no appreciable variation from normal values. Both corticotropin and hydrocortisone administered to adrenalectomized rats caused an increased pituitary concentration of MH.

These observations do not support the concept of a rapidly adjusting adrenal cortex-MH axis for the rat.

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EFFECT OF HUMAN CHORIONIC GONADOTROPIN ON THE SECRETION OF TESTOSTERONE AND 4-ANDROSTENE-3,17-DIONE BY THE CANINE TESTIS[1]

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THE isolation of testosterone and 4-androstene-3,17-dione from spermatic venous blood of the dog has been reported by West *et al.* (1), but the effect of gonadotropins on the secretion of these steroids has not been studied. Human chorionic gonadotropin (HCG) causes hypertrophy of interstitial cells of the testis (2). Brady (3) found that HCG increased the incorporation of acetate-C^{14} into testosterone by testes slices from various species.

In this investigation the influence of HCG on the secretion of testosterone and 4-androstene-3,17-dione by the testis of intact anesthetized dogs was measured by the change in rate of output of these androgens into the spermatic venous blood. The prompt increase in secretion would seem to rule out stimulation of enzyme synthesis as the primary mechanism of tropic action.

METHODS

Male mongrel dogs, 18–28 kg. body weight, were anesthetized with 35 mg. sodium pentobarbital per kg. intravenously. A longitudinal incision was made in the left abdomen, and the spermatic artery and vein were exposed. The spermatic vein was then catheterized caudadly with polyethylene tubing of 0.075 to 0.15 cm. diameter. Care was taken not to interfere with the arterial blood supply to the testis. The animal was given 50 mg. of heparin in saline solution intravenously, and blood was allowed to flow continuously thereafter from the catheter into 100 ml. centrifuge tubes which were changed at 30 minute intervals. One control sample of blood was collected first, then HCG[2] was injected intravenously, and collection was continued for 2–3 hours. The amount in each sample ranged from 40 to 85 ml. whole blood. HCG was given either in single doses of from 1 to 110 I.U. per kg. of body weight or as two hour infusions of 200 I.U. per animal. At the end of the experiments the dogs were sacrificed and testes and prostates were examined histologically.

The blood samples were centrifuged immediately, the plasma transferred to a second centrifuge tube, and 1,000-2,000 counts per minute of testosterone-4-C^{14} of high specific

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[2] A.P.L. Chorionic Gonadotropin was generously supplied by the Ayerst Laboratories.

activity were added to each plasma sample. The radioactivity in the isolated and purified testosterone, when compared with that of the original amount added, permitted calculation of the testosterone in the original plasma.

Each sample was now extracted three times with equal volumes of ether. The ether extracts were then chromatographed on Florisil columns prepared according to Eik-Nes *et al.* (4), and the fraction eluted with 20 ml. 10% ethanol in cloroform was used for chemical estimation of the androgens. By this procedure both the non-steroid lipids and the more polar compounds were eliminated. To separate testosterone and 4-androstene-3,17-dione, the Florisil eluates were chromatographed on paper in a system where the running phase was Skellysolve C and the stationary phase 85% methanol in water (C-85) (5). The spots of the chromatograms were localized by a Haines Ultraviolet Scanner (6) and their Rf's compared with those of standard testosterone and 4-androstene-3,17-dione run at the same time. The two androgens were then eluted from the paper with ethanol.

As a final purification, the ethanol eluates from the C-85 chromatograms were further chromatographed on silica gel columns. These columns were prepared with chloroform and the steroid fraction eluted with 6% ethanol in chloroform. The dried steroid fractions were dissolved in accurate volumes of ethanol and read in the Cary Recording Ultraviolet Spectrophotometer, taking advantage of the absorption maximum at 238–240 mμ, characteristic of α,β-unsaturated ketones. The quantitation was carried out on the basis of the optical densities at 230, 240 and 250 mμ, using the correction described by Allen (7). Aliquots of the testosterone samples were then plated on aluminum planchets and counted in the automatic gas flow windowless counter, to determine the recovery of the added testosterone-C^{14}. The original endogenous testosterone in the blood samples was then estimated by correcting the total steroid, determined by the ultraviolet absorbancy, on the basis of the % radioactivity recovered. Since no radioactive 4-androstene-3,17-dione was available, the recovery of testosterone-C^{14} from each individual sample was also used to calculate the original amount of 4-androstene-3,17-dione in the respective samples. The recoveries averaged 68.5%.

RESULTS

A. *Effect of HCG on Secretion of Testosterone and 4-Androstene-3,17-dione*

The secretion of testosterone from the left testis of ten anesthetized dogs not given HCG, varied from less than 2 to 81.4 μg. per hour, the average being 19 μg. per hour. The corresponding values for 4-androstene-3,17-dione ranged from less than 1 to 16 μg. per hour, averaging less than 3 μg. per hour. In two animals a series of blood samples were collected over a $2\frac{1}{2}$ hour period without HCG injection. The values seemed to decrease slightly during the period of collection.

HCG was given intravenously in three different dosages: (a) One I.U. per kg. of body weight (Fig. 1); (b) 8–9 I.U. per kg. of body weight (Fig. 2); (c) 90–110 I.U. per kg. of body weight (Fig. 3). One I.U. of HCG per kg. of body weight did not cause a significant rise in the output of androgens from the testis into the spermatic venous blood. Doses of HCG from 8 to 110 I.U. per kg. of body weight gave a significant rise in the output of androgens. This was evident in the first half hour after injecting HCG. Within 1 hour after HCG injection the androgen output reached a maximal level which was maintained for several hours thereafter.

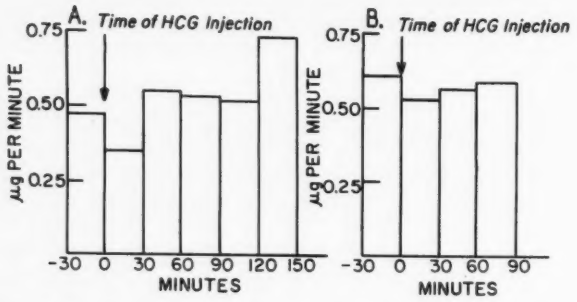

FIG. 1. Changes in the rates of testosterone output in spermatic venous blood of two dogs following injection of one I.U. HCG per kg. body weight. No 4-androstene-3,17-dione was detected.

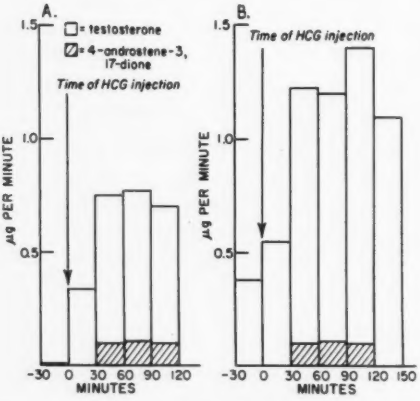

FIG. 2. Changes in the rates of testosterone and 4-androstene-3,17-dione output in spermatic venous blood of two dogs following injection of 9 I.U. HCG per kg. body weight (Fig. 2A) and 8 I.U. HCG per kg. body weight (Fig. 2B). In one experiment (Fig. 2A) the testosterone level before injection of HCG was below the measurable limit. The individual 4-androstene-3,17-dione samples from each time period of both experiments were combined, and both Fig. 2A and 2B show the average values from these experiments. No 4-androstene-3,17-dione could be detected in the first two samples. 4-androstene-3, 17-dione was not measured in the last sample of 2B.

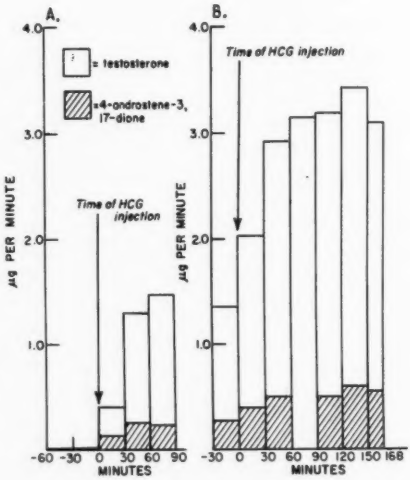

FIG. 3. Changes in the rates of testosterone and 4-androstene-3,17-dione output in spermatic venous blood of two dogs following injection of 110 I.U. HCG per kg. body weight (Fig. 3A) and 90 I.U. HCG per kg. body weight (Fig. 3B). In one experiment (Fig. 3A) the levels of testosterone and 4-androstene-3,17-dione before injection of HCG were below the measurable limits. In the other experiment (Fig. 3B) the fourth sample of 4-androstene-3,17-dione was partially lost and is not included in the figure.

To study this endocrine system further, 200 I.U. of HCG were infused into the femoral vein at a constant rate of 100 I.U. per hour. During this constant infusion of HCG a delay in the response to the hormone was noted in both animals tested (Fig. 4). An increase in the rate of androgen output became evident, however, after 2–4 I.U. of HCG per kg. of body weight had been infused. High levels of androgens were maintained in the

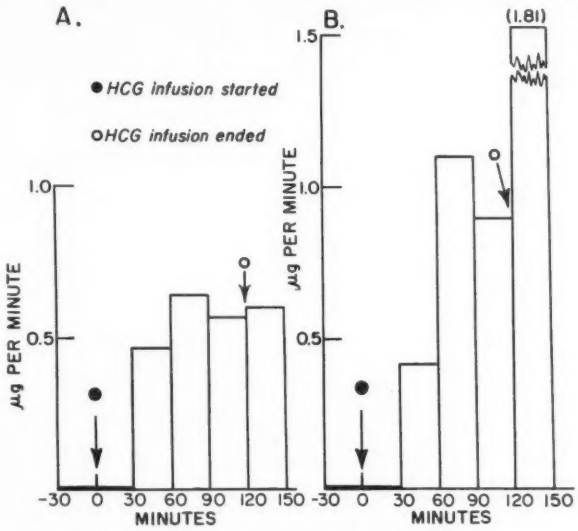

FIG. 4. Changes in the rates of testosterone output in spermatic venous blood of two dogs during constant infusion of 200 I.U. HCG per animal. 4-androstene-3,17-dione was not measured.

spermatic venous blood for at least 30 minutes after the infusion had ceased.

Upon histological examination, all animals used were found to be sexually mature.

B. *Identification of Testosterone and 4-Androstene-3,17-dione*

West *et al.* (1) showed conclusively the presence of testosterone and 4-androstene-3,17-dione in spermatic vein plasma of dogs. Their methods included infrared spectrophotometric analysis. Partly because we did not accumulate large enough quantities of testosterone and 4-androstene-3,17-dione to carry out recrystallizations, and partly because the work of West *et al.* (1) had proved that these compounds were present in dog spermatic vein blood, we turned to less elaborate methods utilizing chromatographic systems and radioactive isotope studies. Both testosterone and 4-androstene-3,17-dione were chromatographed in C-85 and BC-80 systems. The

BC-80 system utilizes 1 part benzene to 2 parts Skellysolve C as the running phase and 80% methanol in water as the stationary phase. In each system the presumed testosterone and 4-androstene-3,17-dione from the spermatic venous blood ran with the same R_f as an authentic sample.

When treated with pyridine and acetic anhydride overnight at room temperature and rechromatographed in the C-85 system, the presumed 4-androstene-3,17-dione spot ran with the same R_f as before, while that which appeared to be testosterone ran with an R_f identical with that of testosterone acetate. There was no trace of 17α-hydroxyprogesterone since all ultraviolet absorbing material with R_f similar to that of testosterone moved after acetylation.

Since testosterone-4-C^{14} had been added to the blood samples after their collection, this also served as an internal standard. All the radioactivity originally present in the free testosterone could be accounted for in the acetylated testosterone. The specific activity of the free testosterone was the same as that of acetylated steroid. Even when both spots were divided into a more rapidly moving half and a slower moving half, the specific activities of all four portions were the same. The measurement of specific activity in different areas of a chromatographed spot has been suggested as a tool for determining if an unknown substance has an R_f identical with that of the added isotopic compound (8). Where the two are the same, the radioactivity will be evenly distributed in the unlabeled compound, and the specific activity will be the same throughout. When the same is the case for a derivative, even stronger evidence for the chemical identity of the carrier and isotopic compound is obtained. The data presented, therefore, give good evidence for the presence of testosterone, since the R_f's of both the free and acetylated forms were found by this method to be identical with those of standard testosterone-4-C^{14} and testosterone-4-C^{14} acetate respectively.

The evidence for the presence of 4-androstene-3,17-dione is good, although not as strong as in the case of testosterone. 4-Androstene-3,17-dione from the spermatic vein blood was chromatographed in two chromatographic systems and ran with the same R_f as that of standard 4-androstene-3,17-dione. An effort to acetylate the compound failed, since after treatment with pyridine and acetic anhydride it still ran with the same R_f. Thus no unhindered alcohol group was present. The only compounds having an α,β-unsaturated system and no unhindered alcohol groups which would show these characteristics are unsaturated diketones. In view of the previous identification of 4-androstene-3,17-dione in dog spermatic vein blood, this would seem to be the compound measured in our experiments.

No radioactivity could be detected in the 4-androstene-3,17-dione spot. This shows that testosterone was not oxidized to 4-androstene-3,17-dione during the analytical procedures. The latter compound must, therefore, have been present in the original plasma.

DISCUSSION

The rapid increase in the output of androgenic hormone following HCG stimulation can be the result of either a release of hormone or an increased synthesis of the hormone. It is likely, however, that HCG enhances the increased synthesis of the hormone since the amounts of testosterone which have been isolated from testicular tissue are quite low (9). In one of our experiments we isolated a total of 0.275 mg. testosterone from the blood collected from one testis under HCG stimulation during 189 minutes. On the basis of isotope recovered, the actual amount present was 0.525 mg. This is considerably more than one would expect to find if a single dog testis was extracted. Another tropic hormone, ACTH, has been shown by Haynes and Berthet (10) to enhance synthesis of corticosteroid rather than to increase release. The situation with HCG appears analogous.

Increased biosynthesis involves changes in enzyme systems. The question arises whether this effect is due to formation of enzyme from precursors present in the interstitial tissue of the testes or whether HCG acts on an enzyme already present. Samuels and Helmreich (11) found that HCG increased the formation of one of the enzymes in the sequence of androgen biosynthesis, the steroid-3β-ol-dehydrogenase. They indicated, however, that this might be an indirect effect associated with a general increase in protein mass, demonstrated by the hyperplasia of the organ following HCG stimulation, since significant increases only occurred 12–24 hours after injection. This change is not compatible with the rapid increase in androgen output following HCG stimulation, and it is, therefore, considered most probable that HCG acts on an enzyme already present in the interstitial tissue. The present data do not rule out completely, however, the possibility that HCG might accelerate the formation of the active enzyme through simple splitting, such as the conversion of pepsinogen to pepsin or the conversion of trypsinogen to trypsin, by which the enzyme would be formed rapidly.

If HCG activates an enzyme system, the next step would be to find the specific reaction most likely to be affected by this tropic hormone. The following scheme has been previously suggested for the biosynthesis of testosterone (12): 5-pregnene-3β-ol-20-one$\rightarrow$progesterone$\rightarrow$17α-hydroxyprogesterone$\rightarrow$4-androstene-3, 17-dione$\rightarrow$testosterone. Of these compounds only testosterone and 4-androstene-3,17-dione have been found in the spermatic venous blood, indicating that the others are either very rapidly turned over or do not escape from interstitial cells. However, only 5-pregnene-3β-ol-20-one and testosterone have been found in the testis (13, 14). If this is the case, the rate-limiting step does not appear to be the metabolism of either progesterone or 17α-hydroxyprogesterone. It is more likely that the action of HCG might be on the dehydrogenation of 5-pregnene-3β-ol-20-one or on a step previous to this reaction. Action on the dehydrogenation step appears unlikely because of the findings that HCG did not

stimulate the 3β-ol-dehydrogenase *in vitro* (11). The fact that the ratio between testosterone and 4-androstene-3,17-dione is unchanged during HCG stimulation shows that the site of action is not the conversion of 4-androstene-3,17-dione to testosterone. It seems, therefore, that the action is either on an early step in synthesis or on a reaction supplying energy to a number of the biosynthetic enzyme systems. It has been suggested by Haynes and Berthet (10) that corticosteroid synthesis in the beef adrenal is dependent on the rate of production of reduced triphosphopyridine (TPN). They found that ACTH causes a rapid activation of phosphorylase in adrenal tissue, thereby increasing the synthesis of glucose-1-phosphate. Glucose-1-phosphate is rapidly converted to glucose-6-phosphate which, by dehydrogenase action, causes the formation of reduced TPN. More reduced TPN in turn increases the synthesis of corticosteroids through mass action effect in the hydroxylation reactions.

HCG was found to cause increased androgen secretion as long as 3 hours following a single injection. This might be expected since HCG is destroyed and excreted slowly. Following intramuscular doses of 4,000 to 10,000 I.U. of HCG in man, detectable amounts of the hormone were found in the urine 4–5 days later (15, 16). Pregnant women have been found to have detectable amounts of biologically active HCG in the blood up to 9 hours after parturition (17). The delay in response during constant infusion until 2–4 I.U. HCG per kg. body weight had been infused can best be explained as due to the accumulation of the minimal concentration of hormone necessary to produce a measurable increase in androgen secretion. Since this is approximately the minimal level effective in a single injection, only slow destruction is again indicated.

Our experiments indicate that the primary effect of HCG is to cause an immediate increase in the rate of androgen secretion, and that increased protein synthesis and enlargement of the interstitial tissue of the testis may be a secondary effect. It is probable, however, that the latter also leads to increased androgen output. Although the results do not permit any final conclusions as to the mechanism of action of HCG, it is felt that this hormone activates an enzyme system related to the biosynthesis of androgens.

SUMMARY

Within the first 30 minutes after the injection of human chorionic gonadotropin the levels of testosterone and 4-androstene-3,17-dione in the spermatic venous blood of dogs were markedly increased. The minimum doses of HCG necessary to produce this effect were between 1 and 8 I.U. per kilogram body weight. Following a single injection of HCG, high levels of these androgens in the spermatic venous blood were maintained for several hours. The primary action of HCG in our experiments is probably to cause an increased output of androgenic hormones by the testicular tissue.

Increased protein synthesis as demonstrated by hyperplasia of this tissue, may be a secondary HCG effect.

Acknowledgments

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THE ADRENAL FETAL ZONE: ITS OCCURRENCE IN PRIMATES AND A POSSIBLE RELATIONSHIP TO CHORIONIC GONADOTROPIN[1]

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THE human fetal adrenal gland is proportionately by weight ten to twenty times larger than the adult gland. It is composed largely of a histologically distinct reticular zone which involutes by necrosis after birth; this zone is known as the fetal zone. Neither the function of the fetal zone nor the tropic factor causing its growth are known (1). No common laboratory animal has a similar structure, and extensive investigation of other mammals has revealed only a few which appear to follow the human pattern. Almost all of these are primates. Hill (2, 3) has found evidence of a fetal zone in two species of lemurs (a primitive primate suborder), the macaque, and a Ceylonese monkey. Uotila (4) found that the fetal zone appeared in the 12.8 mm. macaque fetus, its appearance preceded that of the adult-type cortex. The only non-primates reported to have a fetal zone are the leopard, and possibly the lion and tiger, reported by Hill (5).

The unusual phylogenetic distribution of fetal zone tissue suggested that studies in comparative anatomy of primates might help explain its occurrence and perhaps provide an experimental animal for further work. With the help of many cooperating donors, specimens of neonatal primates from several species have been collected. Wide variation was found in the adrenals of newborn primates, some having a fetal zone and others not. In the macaque the fetal zone disappeared before birth by transition into adult-type cortex. There is evidence that chorionic gonadotropin may be responsible for the growth of the primate fetal zone.

METHODS

Formalin fixed specimens of either whole animals or their adrenals were sent by various cooperating donors, together with the animal's age at death and adrenal and body weights. The adrenals were stained after frozen section with sudan III for fat and after paraffin imbedding with hematoxylin and eosin. Criteria for evaluating the presence of a fetal zone were: 1) histologic examination for a well demarcated central adrenal cortical

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reticular zone resembling that found in the newborn human. The human fetal zone at birth makes up about 80% of the cortex and contains large cells with abundant eosinophilic cytoplasm and large, round nuclei. The irregular nuclear chromatin network stains with moderate intensity. The fetal zone at birth contains only small amounts of fat staining material. 2) The adrenal/total body weight ratio, using figures from neonatal (6) and adult (7) humans for comparison: 3) evidence of adrenal involution. The human gland decreases about 50% in absolute weight during the first three weeks of postnatal life, and

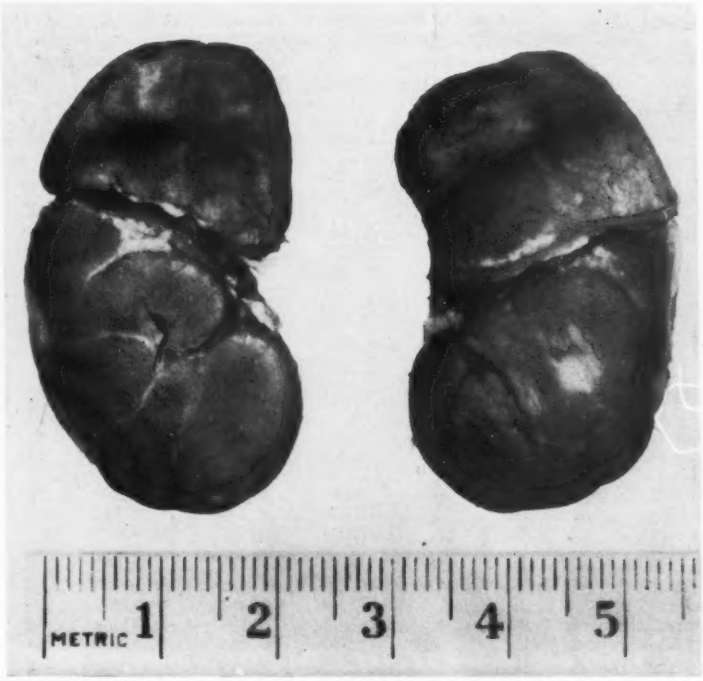


FIG. 1. Kidneys and adrenals from a 4-day old chimpanzee. The adrenals were more than $\frac{1}{3}$ the size of the kidneys.

vascular dilatation, hemorrhage, and necrosis appear in the fetal zone. As the cellular debris is removed, the collapse of the supporting fibrous stroma forms a paramedullary fibrous zone which disappears after about a year. Increasing amounts of fat-staining material appear in the fetal zone within a few days after birth, beginning centrally and extending toward the outer surface. By 10 or 15 days after birth, the entire fetal zone stains intensely for fat.

RESULTS

The Chimpanzee: One 8-year old adult, one 2-day old, and one 4-day old chimpanzee were examined. The two neonatal adrenal glands resembled each other closely. Both were large and lay on a flattened upper pole of the kidney (Fig. 1). The weight of the adrenals relative to body weight closely paralleled those found in the human newborn infant (Table 1, Fig. 2). On

section, (Fig. 4a) the infant chimpanzee adrenals had a thin glomerular and a short fascicular zone. A thick central reticular zone making up about 75% of the cortical radius showed vascular engorgement, hemorrhage, pyknotic nuclei, and necrosis at the central part of the gland. The reticular cells when intact were large, with pink-staining cytoplasm and large round nuclei. Fat stain revealed moderate amounts of fat in the central half of the

TABLE 1. ADRENAL AND TOTAL BODY WEIGHTS AND THEIR RATIOS AS RELATED TO THE HISTOLOGICAL PRESENCE OR ABSENCE OF A FETAL ZONE

Species	Age (days)	Weight (gms.)		Adrenal wt./body wt. ratio	Histologic evidence of a fetal zone
		Total body	Adrenals (pair)		
Human	Newborn[a]	3,439	9.3	0.0027	+
	Adult[b]	60,400	11.6	0.00019	−
Chimpanzee	2	1,500	4.2	0.0028	+
	4	1,500	3.4	0.0022	+
	Adult	26,400	6.0	0.00023	−
Macaque	105* (fetus)	54	0.13	0.0024	+
	110* (fetus)	79	0.11	0.0014	+
	2	197	0.32	0.0016	±
	5	350	0.21	0.00060	−
	17	410	0.26	0.00060	−
	Adult[c]	3,505	0.91	0.00026	−
Congo Potto	Newborn	32	0.087	0.0027	±
	1	30	0.065	0.0022	±
	2	36	0.12	0.0033	±
	4	38	0.077	0.0020	±
	11	30	0.040	0.0013	±
	Adult	1,136	0.13	0.00011	−
Miscellaneous Primates					
Slow Loris	Newborn	30	0.069	0.0023	−
Marmoset	Stillborn	8	0.021	0.0026	+
Cercopithecus Hybrid	Stillborn	240	0.14	0.00058	+
Cercopithecus Hybrid	1	200	0.053	0.0026	−
Cercopithecus Hybrid	12	?	0.096	—	−
Angola Colobus	10	8	0.021	0.0026	+

* Gestation Age.
[a] Reference 6.
[b] Reference 7.
[c] Reference 9.

cortex and less in the outer half. These findings are identical with those seen in the human fetal adrenal in an early stage of involution.

The adult chimpanzee adrenal (Fig 4b) had a glomerular and thick fascicular zone. No reticular zone was seen, but centrally there was a thin paramedullary fibrous zone. Fat staining was intense throughout the gland. The adrenal/body weight ratio resembled that of the human adult.

The Macaque (*macaca mulatta*). Two fetal macaques of 105 and 110 days gestational age (average gestation period: 164 days (8)), 3 infants age 2, 5, and 17 days, and one adult were examined. Birth weights of the infants were 197, 350, and 410 gms. The normal range of birth weight is 330 to

600 gm. with a mean of 435 gm. (8), indicating that the 2-day old infant was premature. This infant died of unknown causes; the others were sacrificed while presumably healthy.

The fetal macaques had large adrenals, (Table 1, Fig. 2) with thick central reticular zones made up of large, eosinophilic cells (Fig. 5a). These were entirely similar to the human fetal zone (Fig. 3a). The two-day old

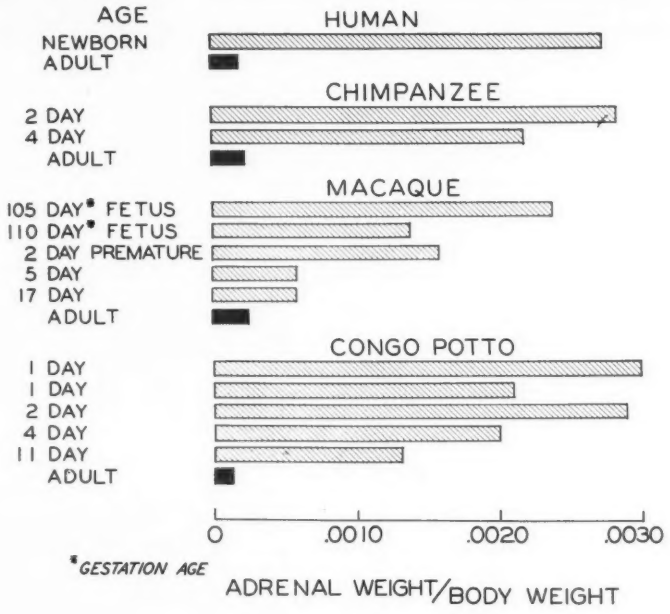


FIG. 2

premature infant had a thick, poorly delimited, central reticular zone composed of somewhat smaller cells, faintly eosinophilic and with large but irregular nuclei. Severe vascular engorgement was present. The 5- and 17-day old infants had poorly delimited central reticular zones with small, basophilic cells and moderate vascular engorement (Fig. 5b). These bore little resemblance to the human fetal zone. The adult macaque adrenal had a central reticular zone merging without change in cell type with the fascicular zone. It was composed of still smaller cells with highly vacuolated, faintly eosinophilic cytoplasm and small, irregular nuclei.

The Congo Potto (*Perodicticus potto*). Five specimens were obtained. One died on the day of birth; others were age 1, 2, 4, and 11 days, and one was adult. The adrenal/body weight ratio and the absolute and relative postnatal diminution of adrenal weight resembled the findings in the human newborn (Table 1, Fig. 2). Adrenal glands from each of the 5 infant pottos (Fig. 6a) had very thick central reticular zones with striking vascular en-

gorgement, little or no fat staining material, and some necrosis. The reticular cells were small and had small, intensely stained nuclei. The weight ratios and thick reticular zone of the neonatal potto adrenals resembled the findings in the human newborn infant, but the cellular morphology in the reticular zone of all the potto adrenals and the small amount of fat in the 11-day specimen were dissimilar.

The adult potto adrenal had a glomerular zone sharply delineated from a fascicular zone (Fig. 6b). The latter contained large, highly vacuolated cells with small, dark, round nuclei. More centrally the cells became smaller and less vacuolated. There was no resemblance to a fetal zone.

Miscellaneous other primates (*Table 1*). Other neonatal primates with histological evidence of a fetal zone were a stillborn African hybrid (*Cercopithecus neglectus*×*Cerçopithecus mona wolfi*) and a stillborn black-tailed marmoset (*Callithrix argentata*).

Those without histological evidence of a fetal zone were two African hybrids (*Cercopithecus l'hoesti*×*Cercopithecus hamlyni*, 1-day old; *Cercopithecus l'hoesti*×unknown African monkey, 12-day old), a newborn slow loris, and a 10-day old colobus monkey (*Colobus polykomos angolensis*). The magnitude of the adrenal/body weight ratios (Table 1) did not always correlate with the presence or absence of a fetal zone.

FIG. 3a. Human neonatal adrenal. The thin basophilic outer zone contains the adult-type glomerular and fascicular zones. The thick eosinophilic zone extending to the central veins is the fetal cortex. Only scanty medullary tissue is present in the human neonatal adrenal; none is visible in this section. ×36.

FIG. 3b. Human adult adrenal. The gland is divided into the glomerular, fascicular, and reticular zones. The reticular zone is much less prominent than in the neonatal gland. ×36.

FIG. 4a. Two-day chimpanzee. A glomerular and short fascicular zone are present. Centrally, a thick, eosinophilic reticular zone with some involutionary changes is present and closely resembles the human neonatal gland during early involution. ×36.

FIG. 4b. Adult chimpanzee. The cortex closely resembles that of the human adult adrenal. A thick medullary zone surrounds the central vein.

FIG. 5a. Fetal macaque. This section closely resembles that of the neonatal human adrenal. ×36.

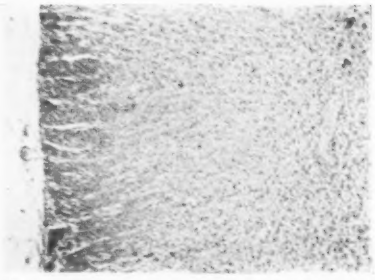
FIG. 5b. Newborn macaque. The cortical cells are small and predominately basophilic. Nothing resembling a fetal zone persists, but the gland has not yet reached the adult configuration, in which the zones more nearly resemble those in the human or chimpanzee adult. ×36.

FIG. 6a. Newborn Congo potto. A thin glomerular and fascicular zone lies at the periphery. Centrally, a thick reticular zone with severe vascular engorgement and some necrosis resembles an involuting human fetal zone. ×36.

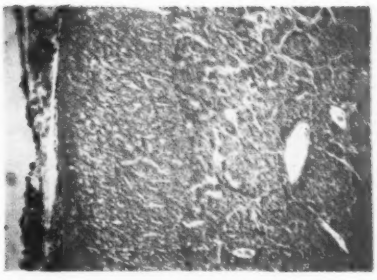
FIG. 6b. Adult Congo potto. The gland contains the usual divisions into glomerular, fascicular, and reticular zones; the last is not prominent. A large central medullary zone is present. ×36.

3a

3b



4a



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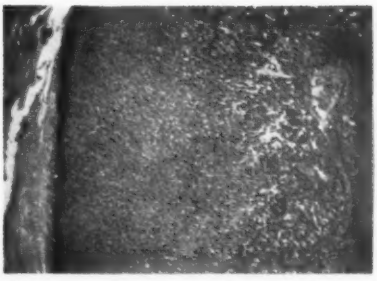
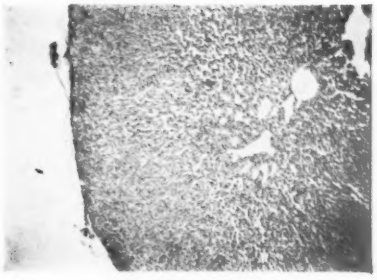


5a



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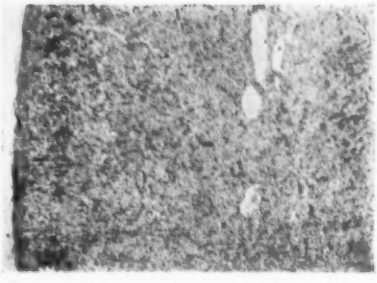
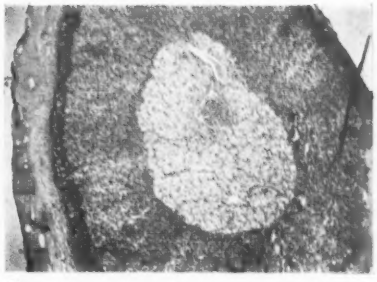


6a



6b

DISCUSSION

The present study has revealed a wide variability in the structure of the neonatal primate adrenal. The newborn chimpanzee has a fetal zone indistinguishable from that of man. The Congo potto has a large adrenal which involutes postnatally, although histologically dissimilar in some ways from the human gland. Certain newborn African monkeys and a slow loris have no evidence of a fetal zone.

Observations in the macaque afford an explanation for these differences. A normal, non-involuting fetal zone was found in fetal macaques two months before term but not in full-term newborns, indicating that the fetal zone disappears before birth in this animal. However, the glands of the full term animals revealed no trace of a previously existing fetal zone. Active involution is evident in the human gland for three or four months, and a residual paramedullary fibrous zone persists for a year or more. Massive fetal zone necrosis could scarcely have occurred in the late fetal macaque without some evidence persisting in the neonatal gland. It seems likely that the fetal zone in this animal disappears by transition into adult type cells and not by necrosis. This concept was supported by finding an intermediate cell type in the adrenal of a prematurely born macaque.

Prenatal disappearance of the fetal zone in some species may explain the unexpectedly diverse findings in neonatal primates. Absence of a fetal zone in the newborn animal might indicate only that it has disappeared previously; the morphology of the gland during fetal life must be known before concluding that a fetal zone is absent. Opportunities for such observations are almost non-existent for most primates.

Disappearance of the fetal zone cells by transition into adult type cells suggests a functional relationship between the two cell types. Their different appearance does not preclude this relationship. Symington and coworkers (10) have induced striking morphologic changes in the adult human adrenal by ACTH administration; the transition of adult-type reticular zone cells into large, fat depleted, eosinophilic cells resembling those of the human fetal zone was associated with evidence of increased steroidogenic activity and histochemical evidence of increased amounts of ribonucleic acid and certain intracellular enzymes. Some similar tropic stimulus must produce the adrenal hypertrophy seen in the primate fetus. This stimulus must be peculiar to primates during gestation and must show species differences corresponding to the differences in fetal zone development. A hormone meeting these requirements is chorionic gonadotropin (CG), previously suggested by Jones (11) as a possible trophic hormone for the fetal zone. This hormone is made in the primate placenta and is not found in other mammalian orders. Data on CG excretion during pregnancy in man, the chimpanzee, and the macaque are available. In man (12) the urinary titer of CG during pregnancy rises abruptly, beginning about the 40th day after the last menstrual period and reaching a high

peak at about 70 days. By 110 days it falls to a low level which is maintained throughout the rest of pregnancy but which is too low in some individuals to yield a positive Ascheim-Zondek (A-Z) test late in pregnancy. The fetal zone reaches its maximum relative size at 60–120 days (13, 14). The chimpanzee (15, 16) has a shorter gestation period (230 days) and a proportionately shorter period of urinary CG excretion. The A-Z test is regularly positive in the period 61–90 days after conception, with extremes of 25–157 days. The macaque (17) has a disproportionately brief period of significant CG excretion in early pregnancy; the A-Z test is positive in the period 19–25 days after ovulation; the period of gestation is about 164 days. The macaque is the weakest producer of CG and has a fetal zone which involutes before term. Man and the chimpanzee are both more potent producers of CG and both maintain a fetal zone until birth. The data are consistent with an influence of CG on the fetal zone.

CG might act directly on the fetal adrenal to produce hypertrophy or it might act indirectly by influencing the intensity of the adrenotropic stimulus from the fetal pituitary. The latter appears more likely. Anencephalic monsters, in which the fetal pituitary is severely defective, are born with hypoplastic adrenals containing little or no fetal zone, and a similar lesion has been found in the adrenals of several newborn infants born of cortisone-treated mothers. These findings indicate that a tropic stimulus from the fetal pituitary is necessary for the maintenance of a fetal zone and that this stimulus can be inhibited by cortisone in the same fashion as in the postnatal infant or adult. CG might influence this stimulus in several ways. It might impair the adrenal production or accelerate the peripheral utilization of those adrenal corticosteroids capable of suppressing pituitary adrenotropin production. It might stimulate the production of estrogens, which in turn are believed to promote pituitary adrenotropin release. And it might act directly on the fetal pituitary. Further work testing these possible relationships is in progress.

SUMMARY

A fetal zone was found in the adrenal glands of some newborn primates (chimpanzee, Congo potto, marmoset) but not others (cercopithecus hybrid monkeys, slow loris).

A fetal zone was found during fetal life in the macaque, this zone disappeared before birth by transition into adult-type cortex. This finding affords an explanation for the unexpected variations in the adrenals of various newborn primates. It also suggests a functional relationship between fetal zone cells and those of the adult cortex.

There is evidence that chorionic gonadotropin is responsible for the development and maintenance of the fetal zone, probably by influencing the intensity of the adrenotropic stimulus from the fetal pituitary.

Acknowledgments

These observations were made possible by the kind cooperation of Dr. Leonard J. Goss and Dr. Charles Gandal, of the New York Zoological Society; Dr. Glen Crosbie, of the Zoological Society of San Diego; Dr. Henry Nissen, of the Yerkes Laboratory for Primate Biology, Orange Park, Florida; and Dr. Gertrude Van Wagenen, of the School of Medicine, Yale University, New Haven. Dr. Donald Davis very kindly removed an adrenal from the adult Congo potto, making it unnecessary to sacrifice this interesting animal.

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ADRENAL REGENERATION HYPERTENSION[1]

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SKELTON (1–4) has reported that hypertension, associated with characteristic pathologic lesions, develops if one adrenal is removed and the other enucleated and allowed to regenerate in rats sensitized by unilateral nephrectomy and a high sodium chloride intake.

The studies reported here were undertaken to determine if we could confirm Skelton's reports, and if so, to examine the effects of various endocrine and pharmacologic manipulations in enucleate rats. Our results show unequivocally the reality of the syndrome described by Skelton and demonstrate its responsiveness to various treatments. An abstract of this work has been published (5).

METHODS

Most of our experiments were performed on female rats of about 150 gm. initial body weight. In addition, observations were made on males of the same size and small females (ca. 58 gm.). One kidney was removed in all cases and 1% sodium chloride substituted for drinking water. This procedure sensitizes animals to the pressor influence of regenerating adrenals as it does in other types of hypertension (1–4). Except in controls (Group I), the right adrenal was removed and the left enucleated by gently lifting the gland, nicking the capsule, and squeezing out the medulla and most of the cortex. The animals were observed for 7 weeks (except in the amphenone series) and then autopsied.

Drugs were given daily 6 times per week as follows: hydrocortisone (free alcohol) in aqueous suspension subcutaneously; reserpine (Serpasil) subcutaneously; amphenone B by stomach tube. Hydralazine (Apresoline) was given in the drinking fluid with concentrations adjusted to obtain intakes approximating the tabulated dosage. A group of untreated adrenal enucleate animals was observed simultaneously in all cases with treated groups. These controls are all grouped together in the table and figures.

Blood pressure was determined without application of external heat or anesthesia, using a photoelectric tensometer (Metro Industries). The blood pressure values for most groups could be represented as straight lines between the third and sixth week only and are so indicated within 95% confidence limits in Figures 1–4. Figures were incomplete and irregular for the earlier weeks and variable during the last week of observation. The validity of the straight-line relationships involved in the groups treated chronically was established by Analyses of Variance and F tests for all groups except those treated with 1 mg. of hydrocortisone. The limits were based on the standard errors of the lines, the coefficient chosen for 5% level of significance and the degrees of freedom established

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[1] The authors are indebted to Dr. J. H. Leathem for evaiuation of the histological preparations obtained in this work and to Mrs. Hanna Sylwestrowicz for statistical analysis of the data.

for each compound by Analyses of Variance (6). Analysis of the data shown in Table 1 was based on Analyses of Variance and a Modified Tukey Test used to locate differences between groups at 5% levels of significance (7).

RESULTS

Controls. The control pre-treatment blood pressure of 79 animals from this series was 115.8 ±9.8 (Stan. Dev.) mm. Hg. The standard error of this value was ±1.1 and the extreme range for single readings 89 to 145.

Blood pressure rose mildly but distinctly in animals subjected only to the sensitizing procedure, i.e., unilateral nephrectomy and 1% sodium chloride in drinking fluid (Fig. 1; Table 1, Group I). This response, however, did not approach in magnitude that observed in most of the animals which in addition had unilateral adrenalectomy and unilateral adrenal enucleation. Furthermore, the blood pressure in the control group did not rise progressively, as in the enucleate animals, during the third to sixth week.

Adrenal Enucleation (Fig. 1; Table 1, Group II). The experiments were generally run on groups of 6 animals at intervals over a period of 12 months. The Skelton procedure caused marked hypertension in every one of the several groups with only an occasional individual exception. The syndrome was of such severity as to be the apparent cause of death in 18% of the cases within the 7-week period of observation. The irregularity of values obtained during the seventh week (see Methods) suggests the possibility that blood pressure may be reaching a plateau or possibly subsiding by that time (cf. Skelton, 1).

The syndrome seemed to develop with the same intensity when male rats (Table 1, Group VIII) or small females (Table 1, Group VII) were used. We have not studied the effects of these procedures on rats as large as those used by Skelton; he reports that large animals are relatively refractory to the treatment (2).

Pathological Changes. No comprehensive study was made of the pathology associated with adrenal regeneration hypertension such as that conducted by Skelton (4). It was evident, however, from gross observation that there was less macroscopic abnormality than previously seen in DCA-hypertension (10). Gross periarteritis nodosa, for instance, was evident in only 2 cases of our untreated animals with adrenal enucleation (Group II) at 7 weeks, whereas it was almost invariably observed at 8 weeks in DCA-treated rats (10).

Systematic histological study was done only on kidney tissue. About 75% of the untreated series (Group II) showed some abnormalities, varying greatly as to intensity and amounts of tissue involved. This consisted, in varying combinations, of nephrosclerosis, nephrosis, casts, round cell infiltration, enlarged glomeruli, tubular ischemia and dilatation and capsular fibrinoid. Again, such changes were less frequent and severe but not eliminated in groups treated with either hydrocortisone or reserpine.

It is not known to what extent the protective effect of these two substances was due to the reduced salt appetite which they induced.

Hydrocortisone (Fig. 2; Table 1, Groups III and IV). Hydrocortisone was given to see if its suppressive effects on the regeneration of the enucleated adrenal would modify the hypertensive syndrome. The larger (1 mg./day) dose inhibited adrenal regeneration markedly. We were aware to begin with that any result in this series would be complicated by the fact

TABLE 1. RESPONSE OF RATS TO ADRENAL ENUCLEATION AND OTHER TREATMENTS

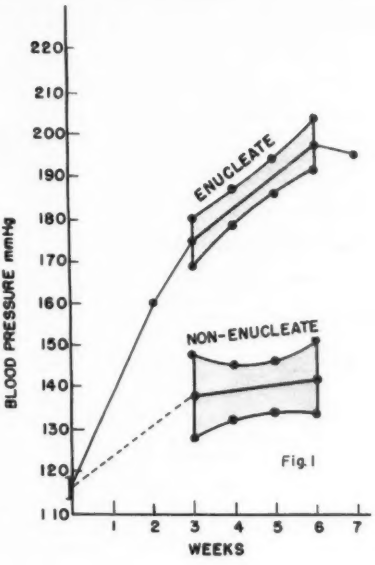
Group	Treatment	No.	Sex	Body wt.		Blood pressure, mm. Hg.		Saline intake, ml./day	Organ wt., mg./100 gm.				% died
				Init.	Term.	3rd wk.	7th wk.		Regen. adrenal	Heart	Brain	Kidney	
I	Uninephrectomy+NaCl	18	♀	140	205	Fig. 1		94	29.7[1]	396*	718	685	5.5
II	Adrenal Enucleate[2]	44	♀	155	201	Fig. 1		109	22.9	457	882	847	13.5
III	Adrenal Enucleate+1 mg. Hydrocortisone/day	21	♀	151	194	Fig. 2		78*	13.4*	422	823	753	9.5
IV	Adrenal Enucleate+0.1 mg. Hydrocortisone/day	12	♀	150	218	Fig. 2		104	17.6	458	855	818	25.0
V	Adrenal Enucleate+10 mg./kg. Hydralazine/day	18	♀	149	187	Fig. 3		114	22.0	544	798	961	39.0
VI	Adrenal Enucleate+100 μg./kg. Reserpine/day	12	♀	155	193	Fig. 4		75*	23.1	451	923	757	8.3
VII	Adrenal Enucleate	12	♀	59	160	146	185	70	20.3	452	857	883	91.
VIII	Adrenal Enucleate	12	♂	152	261	172	187	108	13.8	425	631	787	16.6
IX[3]	Adrenal Enucleate+100 mg./kg. Amphenone B/day	9	♂	161	196	177	—	81	14.7[4]	—	—	845	11.1

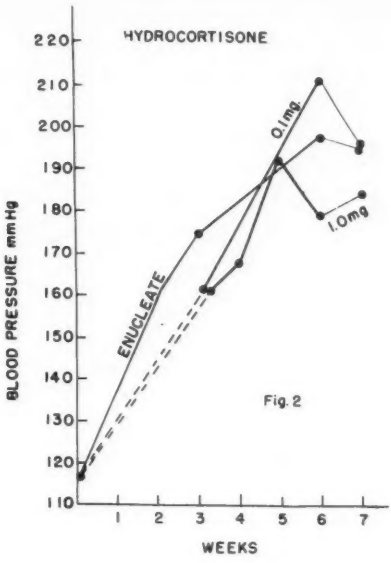
* Significantly different from Group II.
[1] Weight of 2 normal adrenals.
[2] Adrenal enucleation, uniadrenalectomy, uninephrectomy and NaCl.
[3] Treated and followed only 3 weeks.
[4] Adrenal weight of enucleate controls not treated with amphenone: 9.9 mg./100 gm

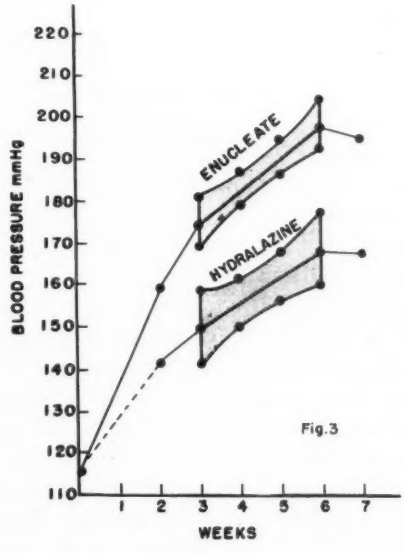
that hydrocortisone, while inhibiting adrenal regeneration, was itself a hypertensive agent (8, 9). An additional complication was that the blood pressure rise during the third to sixth week could not be expressed as a straight line. This was in contrast to results with other treatments. In each of the three groups of animals given 1 mg./day of hydrocortisone, the average curve of which is shown in Figure 2, a definite peak in blood pressure occurred at about the fifth week. Furthermore, the groups treated with the larger dose of hydrocortisone unexpectedly drank less saline than enucleate controls. Whatever may be the meaning of these apparent aberrations, it is clear in general that hydrocortisone provided no substantial protection against the effects of adrenal enucleation.[2] Smaller and presumably non-hypertensive doses of hydrocortisone (0.1 mg./day) caused only slight adrenal suppression of questionable significance and clearly no suppression of the hypertension.

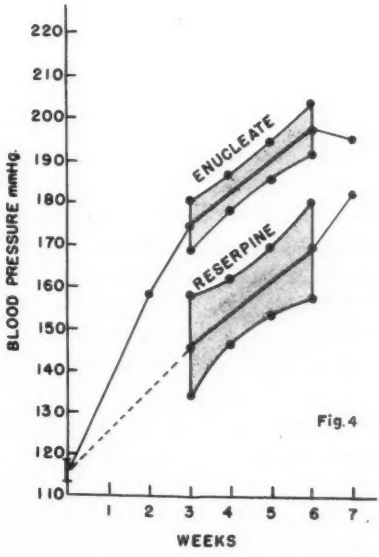
Hydralazine (Apresoline) (Fig. 3; Table 1, Group V). When given chronically, hydralazine reduced blood pressure but not to normotensive

[2] Due to a misprint there is a contradictory statement in the previously published abstract of this work (5).









FIGS. 1–4. Showing the effects of various treatments on "adrenal regeneration" hypertension. All groups except the one labelled "non-enucleate" had one adrenal removed and the second enucleated; in addition, without exception, the animals had one kidney removed and received 1% sodium chloride to drink. The shaded bands show the 95% limits of confidence for the periods indicated. Doses and other information are given in Table 1.

levels. This action was not associated with any diminution in salt intake. Hydralazine was, however, somewhat toxic and the mortality of animals receiving this drug was higher than in any other group; it seemed to be less effective than in DCA-hypertension, as judged by previous studies (10).

The hypotensive action of hydralazine was prompt and marked when it was given in single doses subcutaneously to three animals with established hypertension. Mean values are shown in Figure 5.

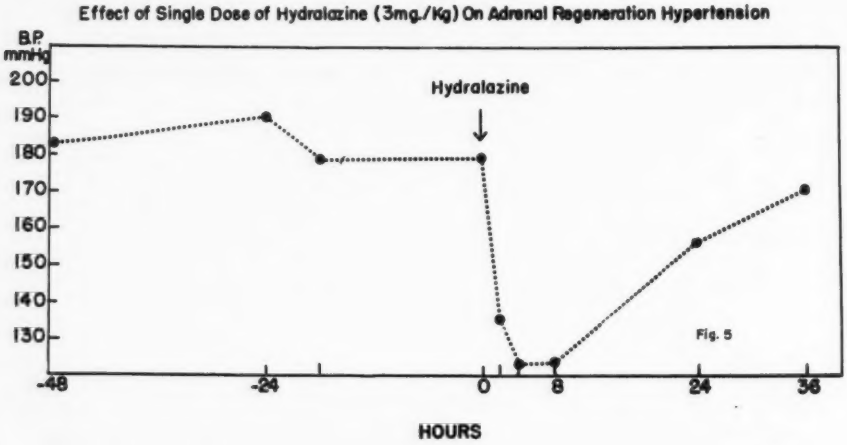


FIG. 5

Reserpine (Serpasil) (Fig. 4; Table 1, Group VI). The chronic administration of reserpine caused a distinct inhibition in the expected rise of blood pressure when results were expressed in mean terms. There were, however, some individual exceptions to this rule in which no apparent protection was provided. The interpretation of results with chronic administration of reserpine was, however, obscured by the fact that this alkaloid, as in DCA-hypertension (10), reduced the voluntary salt intake.

Nevertheless, reserpine can have a dramatic hypotensive action, independent of salt intake, as was illustrated when it was given in a large single dose to animals with established hypertension (Fig. 6). In this experiment each of six animals served as its own control. On one occasion half of the animals received reserpine vehicle and the other half reserpine itself. The following week treatments were reversed. Salt intake was reduced only slightly by this acute treatment but such variation in intake as occurred was controlled by "pair-feeding" of the saline drinking solution. The hypotensive response to reserpine under these conditions was probably as great as has ever been demonstrated in any experimental situation. This does not prove that the effects of reserpine in the chronic experiments were unrelated to the consumption of salt but does show that the alkaloid can act by some less obvious means.

Amphenone B[3] (Table 1, Group IX). It has been demonstrated that amphenone B inhibits adrenal steroid production in the dog (11, 12) and man (13). In man, at least, it may more readily inhibit the production of aldosterone than hydrocortisone (14). In the rat, amphenone causes marked morphological and biochemical changes in the adrenal (15, 16) but except for depressed corticosterone output in *in vitro* incubation studies (17, 18), its action on steroid production, if any, is obscure (19).

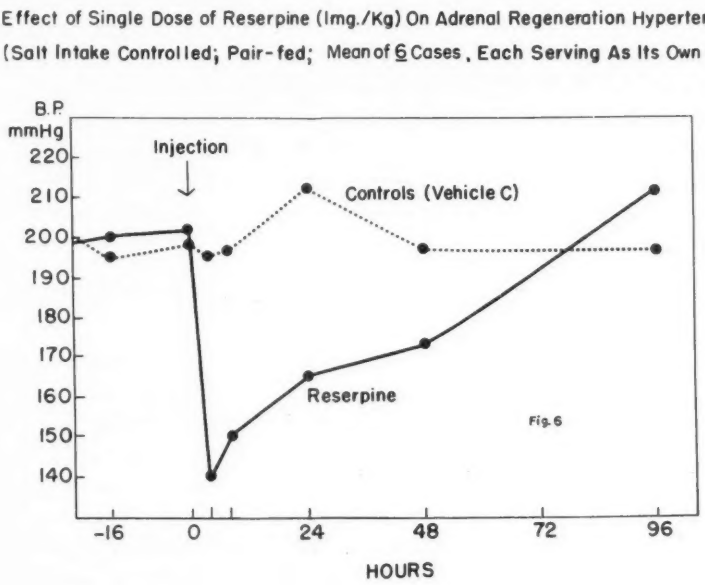


FIG. 6

Several groups of rats were treated with the compound, using various modifications in experimental design. The results from one of these groups, typical of the whole, are shown here (Table 1, Group IX). Male rats were used because most of our experience with other effects of amphenone has been in males. Since amphenone is somewhat toxic, it was given in relatively small doses (100 mg./kg./day) and the experiments were terminated at 3 weeks. Clearly, the compound did not prevent the development of hypertension. It is possible that larger doses might have given a different result, as in the recent experiments of Chappel, *et al.* (29). The amounts used, however, did exert other typical and expected amphenone effects: adrenal hypertrophy (seen in the enucleate gland), thyroid hypertrophy, mild growth inhibition, etc.

[3] Amphenone B was synthesized by Dr. M. J. Allen and associates, Chemical Research Division, CIBA.

DISCUSSION

The discovery that a regenerating adrenal has the ability to cause hypertension was an intriguing one for various reasons, but primarily because it would seem to afford a possible new experimental approach to the elusive problem of the role of the adrenal cortex in hypertension. It was a surprising discovery because of older physiological evidence indicating that enucleate regenerating adrenals were hypofunctional, at least as far as the production of anti-stress hormones (20) and the response to ACTH (21) were concerned.

There are only slight and suggestive distinctions to be made between the adrenal regeneration hypertension as we observed it and DCA-hypertension as previously observed (10). The speed with which the syndrome developed, its response to drugs, and the salt appetite were much the same in the two conditions. The distinction seemed to be that pathological changes, such as periarteritis nodosa, nephrosclerosis, etc., were less severe in adrenal regeneration than in DCA-induced hypertension. We consider such distinctions only suggestive, however, since the two conditions were not studied simultaneously under identical conditions or for the same periods of time.

The possibility has occurred to numerous investigators that regenerating adrenals might secrete relatively large amounts of aldosterone and that this could be the cause of the observed hypertension. This hypothesis is derived by rather devious inference from the following facts: the Greep-Deane theory (22) that the salt-regulating adrenal hormones are produced primarily by the outer glomerulosa layer of the adrenal seems to have been provided with solid objective foundations for both beef (23) and rat (24) glands. In fact, a relative excess production of aldosterone by enucleated rat adrenals *in vitro* was shown by Giroud, *et al.* (24). The question then arises as to whether regenerating adrenals secrete enough aldosterone to cause hypertension, as seems to be the case in human primary aldosteronism (25). It is much too early to suggest any affirmative conclusion on that question. The results reported here with hydrocortisone and amphenone B, while inconclusive in themselves, offer no support for the idea that aldosterone is the causal agent involved. In addition, there is lack of agreement as to the hypertensive actions of aldosterone in rats (26, 27). Certainly, in equal sodium-retaining doses, aldosterone fails to induce hypertension in rats sensitized by uninephrectomy and salt as does DCA (28) or adrenal enucleation. The question can be resolved only by studies yet to be done showing with certainty the pathogenicity of aldosterone and the amounts of this hormone secreted by regenerating adrenals. The fact remains, however, that functionally deranged adrenals, which are hypofunctional by classical criteria, can cause hypertension. A full analysis of the cause of this "adrenal regeneration" hypertension may well contribute eventually

to an understanding of clinical hypertension. The responses of the syndrome to drugs are in general those to be expected in other types of hypertensive disease and do not reveal that it has distinctive traits.

SUMMARY

One adrenal was enucleated and the other removed in rats sensitized by unilateral nephrectomy and a high salt diet. In confirmation of the work of Skelton, this procedure resulted in severe hypertension, associated renal damage and some mortality. The response was not limited by sex nor by size in the range of 60-160 grams body weight. The syndrome resembled that produced by DCA but was accompanied by less pathological damage.

Hydrocortisone, in doses which inhibited adrenal regeneration, did not prevent hypertension but seemed to modify the syndrome in ways not clearly defined, including a reduction of the spontaneous sodium chloride intake.

Hydralazine (Apresoline) reduced blood pressure when given either acutely or chronically but did not decrease salt intake, improve survival or minimize renal damage.

Reserpine reduced blood pressure and saline appetite. It caused marked reduction in blood pressure when given acutely under conditions in which salt intake was controlled.

Amphenone B did not inhibit the hypertensive response to adrenal regeneration.

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STUDIES ON HORMONAL FACTORS INFLUENCING HEPATIC GLUCOSE-6-PHOSPHATASE[1]

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THE behavior of hepatic glucose-6-phosphatase (G-6-Pase) in various physiological and pathological conditions has recently been under investigation in various laboratories. It was shown that in adult rats and mice, liver G-6-Pase activity increased in fasting (2, 3, 4). On the other hand, G-6-Pase activity progressively decreased in the precancerous liver of 4-dimethylaminoazobenzene fed rats and it was almost completely absent in the developed hepatoma. The transplantable Novikoff rat hepatoma and various transplantable mouse tumors showed no G-6-Pase activity (5). Studies in regenerating liver showed that G-6-Pase activity was not affected in the rapidly growing adult liver. On the other hand, almost no enzymatic activity was demonstrable in 16 and 19 day old rat embryos. Higher than normal G-6-Pase activity was found in the liver of newborn rats (6). These changes in the hepatic G-6-Pase activity under different physiological and pathological conditions have focussed our attention upon the factors which may influence the G-6-Pase reaction *in vivo*.

It was reported in previous studies that cortisone administration resulted in hyperglycemic blood sugar levels and highly increased liver G-6-Pase activities in rat (7). Increased blood sugar level and liver G-6-Pase activity after cortisone administration were demonstrated in both fed and 24 hour fasted animals (8). It was suggested that cortisone increased liver G-6-Pase activity and that the increased glucose-6-phosphate (G-6-P) splitting might be responsible, partly at least, for the high blood sugar levels in the cortisone treated animals. The stimulating effect of cortisone on liver G-6-Pase activity raised the possibility that the adrenal gland may play a role in the physiological maintenance and regulation of the activity of this enzyme (8).

In the present paper a comparison is made between blood sugar level and liver G-6-Pase activity in fed and fasted animals after adrenalectomy, hypophysectomy and partial hepatectomy. The results show that a parallelism between blood sugar level and liver G-6-Pase activity can be demonstrated under these circumstances in the fasted animals. This paper also

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[1] A preliminary report of this work was presented at the meeting of the Canadian Physiological Society, London, Ontario, October 13–15, 1955 (1).

[2] Senior Fellow of the Cancer Research Society.

presents evidence showing the significance of the adrenal gland and hypophysis in influencing hepatic G-6-Pase activity.

MATERIALS AND METHODS

Preparation of Animals and Experimental Groups. Young male Wistar rats were used. The animals weighed 100 ± 10 gm. at the beginning of the experiment. The following experimental groups were set up.

(A) 1. Normal, untreated fed rats.
2. Normal, untreated 24-hour fasted rats.
(B) 3. Bilaterally adrenalectomized rats, fed until sacrifice.
4. Bilaterally adrenalectomized rats, 24-hour fasted before sacrifice.
(C) 5. Hypophysectomized rats, fed until sacrifice.
6. Hypophysectomized rats, 24-hour fasted before sacrifice.
(D) 7. Hypophysectomized and partially hepatectomized rats, 24-hour fasted before sacrifice.
8. Hypophysectomized and sham hepatectomized rats, 24-hour fasted before sacrifice.

The adrenalectomies were performed through the lumbar approach under ether anesthesia. The animals received daily 100 μg. of deoxycorticosterone acetate intramuscularly for 3 days and were used for experiments a week after operation. Hypophysectomized rats were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. The completeness of operations was always checked at the autopsy of the animals. Partial hepatectomies (66%) were performed under light ether anesthesia according to the method of Higgins and Anderson (9). Sham operations were performed at the same time under the same conditions. All animals seemed to be in good health at the start of experiment. The animals were maintained on Purina Fox Chow and water *ad libitum*. The fasted animals were placed in individual cages with water available *ad libitum*.

Preparation of Homogenate. The animals were decapitated and blood was obtained from the severed neck vessels for sugar determination. The livers were quickly excised, placed in beakers standing on ice, chilled for 5 minutes, then blotted on filter paper and weighed. The livers were minced with scissors, and 10% homogenates were prepared in 0.25 *M* ice-cold sucrose. Homogenization was carried out with a teflon plastic pestle turning at about 600 r.p.m. for exactly 3 minutes.

Cellularity Determination. The cellularity of the liver was determined by counting the nuclei in the liver homogenate. For this purpose a 10% homogenate was prepared in 0.85% NaCl. An amount of 0.25 ml. of this saline homogenate was taken by a drawn out serological pipette and transferred to a 10 ml. volumetric flask and completed to the mark with staining solution. The staining solution was prepared by dissolving 0.75 gm. of orcein in 200 ml. of a 45% glacial acetic acid solution (10), and it was filtered before use. Samples were taken from the homogenate and placed in an "improved double Neubauer ruling" (Schreck) hemacytometer. Samples were allowed 2 minutes of settling in the counting chamber to avoid movements of nuclei during counting. This way 12 counts were made routinely from a homogenate and the mean value of these determinations was taken.

Biochemical Determinations. Blood sugar was measured by the Folin-Wu method. Nitrogen content was determined by the micro-Kjeldahl procedure. G-6-Pase activity was assayed according to Cori and Cori (11), with slight modification (5), and the release of inorganic phosphate was assayed by the method of Fiske and Subbarow (12). The activity was measured in micrograms of inorganic phosphate released in 15 minutes at 30° C.

Expression of Cellularity and Biochemical Data. The results of cell counts were expressed as "cellularity" (13) indicating the number of liver cells in millions per gram wet weight. The term "total cellularity" introduced here means the number of cells in millions contained in the whole liver. Blood sugar values were expressed as mg. per cent. Nitrogen content was expressed as mg. nitrogen per gm. wet weight, per whole liver (total liver nitrogen) and as total liver nitrogen per 100 gm. body weight. G-6-Pase activity was expressed per unit liver (per wet weight and per average cell) and as total liver G-6-Pase activity per 100 gm. body weight. The total liver G-6-Pase activity per 100 gm. body weight (total available activity) was calculated as follows:

$$\text{Total available activity} = \frac{\text{activity per gm. wet weight} \times \text{liver weight} \times 100}{\text{body weight}}$$

Interpretation of Enzymatic Results. In the interpretation of enzymatic changes the basis on which enzymatic activities are expressed is of importance. G-6-Pase activity was expressed, first of all, per unit wet weight. This was done mainly for the purpose of comparing results with data published in the literature. To counteract the changes in the size of liver, due to alterations in water, glycogen, fat and protein content, the enzymatic activity was expressed per average cell. To express the liver enzymatic activity in relation to the functional need of the whole animal G-6-Pase activity was also expressed as total liver G-6-Pase activity per 100 gm. body weight (total available activity). In comparing the data of normal and experimental groups results were also expressed on a per cent basis when values of the control animals were taken arbitrarily as 100%.

RESULTS

The Effect of Adrenalectomy and Hypophysectomy on Liver Weight, Liver/Body Weight Ratio, Liver Cellularity and Nitrogen Content in Fed Rats. (See Table 1.) Adrenalectomy or hypophysectomy caused a significant decrease in liver weight and liver/body weight ratio. The cellularity and the nitrogen content per gram wet weight were not affected. The total liver cellularity showed an apparent but statistically not significant decrease.

TABLE 1. COMPARISON OF LIVER/BODY WEIGHT RATIOS, CELLULARITY AND NITROGEN CONTENT IN FED AND FASTED RATS UNDER EXPERIMENTAL CONDITIONS[1]

Animals	Liver weight (gm.)	Liver/body weight×100	Cellularity (millions)	Total liver cellularity (millions)	Nitrogen (mg./gm. liver)	Total liver nitrogen (mg.)	Total liver nitrogen per 100 gm. body weight
Fed animals							
Normal*	6.2± .2	4.9± .3	280± 43	1736± 237	32.1± 3.0	194± 8	151± 10
Adrenalectomized	4.5± .7†	3.9± .5	324± 58	1471± 393	32.6± 2.0	148± 29†	129± 21
Hypophysectomized	4.2± .2†	4.0± .1	315± 37	1334± 169	33.0± 3.7	139± 11†	132± 11†
Fasted animals							
Normal*	3.8± .3	3.8± .2	410± 55	1543± 181	36.8± 0.6	141± 13	143± 8
Adrenalectomized	3.5± .5	3.3± .4	321± 48	1114± 262	33.4± 1.8	116± 23	110± 20‡
Hypophysectomized	2.8± .2‡	2.9± .2‡	441± 68	1231± 225	37.3± 1.5	104± 11‡	110± 12‡
Hypophysectomized and sham hepatectomized	2.6± .2‡	3.2± .2‡	326± 39‡	833± 200‡	34.8± 1.8	89± 8‡	111± 7‡
Hypophysectomized and partially hepatectomized	1.1± .2‡	1.3± .2‡	296± 45‡	359± 75‡	30.0± 1.5‡	33± 5‡	39± 4‡

[1] The mean values and standard deviations are given. The means represent six or more animals. The standard deviation = $\sqrt{\Sigma x^2/(n-1)}$. x = deviation from the mean, n = number of animals. Fed: food and water *ad libitum* until sacrificed. Fasted: no food for 24 hours before sacrificed, tap water *ad libitum*.

* Previously published values (8).

† Statistically significant difference as compared to values of normal fed animals ($P = <0.05$).

‡ Statistically significant difference as compared to values of normal fasted animals ($P = <0.05$).

The total liver nitrogen was significantly decreased. The total liver nitrogen per 100 gm. body weight showed a significant decrease only after hypophysectomy.

The Effect of Adrenalectomy and Hypophysectomy on Liver Weight, Liver/Body Weight Ratio, Liver Cellularity and Nitrogen Content in 24, Hour Fasted Rats. (See Table 1). Twenty-four-hour fasting in itself caused marked changes in the liver constituents of normal rats (8, 10). The liver weight decreased by 40% and the liver weight/body weight ratio decreased by 24%. The cellularity and the nitrogen per unit wet weight liver were highly increased. The total number of liver cells did not change significantly during 24-hour fast period, but the total liver nitrogen content was significantly decreased. On the other hand, the total liver nitrogen per 100 gm. body weight was in the range of that of normal fed rats.

Similar types of changes were noticed in adrenalectomized and hypophysectomized animals after 24-hour fasting. The values of the adrenalectomized animals were all lower than those of the normal fasted rats. In the hypophysectomized animals the following values were significantly lower than those in normal fasted rats: liver weight, liver/body weight ratio, cellularity, total liver nitrogen and total liver nitrogen per 100 gm. body weight.

In hypophysectomized animals which were subjected to sham-hepatectomy operation and killed after a 24-hour fast the values were very similar to those of the fasted hypophysectomized animals. In hypophysectomized and partially hepatectomized animals after 1 day fasting all liver constituents were extremely low and, as a result of the operation, ranged about one-third of those of the fasted hypophysectomized animals.

The Effect of Adrenalectomy and Hypophysectomy on Blood Sugar and Liver G-6-Pase Activity in Fed Rats. (See Tables 2 and 3). When food was available *ad libitum* there was no significant difference in the blood sugar level of normal, adrenalectomized or hypophysectomized rats (Table 3).

Liver G-6-Pase activity was apparently not affected in fed adrenalectomized animals when expressed per unit wet weight, but a significant decrease was shown when data were expressed per cell. In fed hypophysectomized animals liver G-6-Pase was significantly decreased per unit wet weight and in the average cell (Table 2). Total activity was decreased to 62–56% of normal values (Table 3) in both adrenalectomized and hypophysectomized animals.

In fed animals a parallelism between blood sugar level and liver G-6-Pase activity cannot be well demonstrated because of the variable food intake and absorption rate. This difficulty is eliminated in fasting animals when liver is the only source of blood sugar.

The Effect of Adrenalectomy and Hypophysectomy on Blood Sugar and Liver G-6-Pase Activity in 24 Hour Fasted Rats. (See Tables 2 and 3). Blood

TABLE 2. COMPARISON OF LIVER GLUCOSE-6-PHOSPHATASE ACTIVITY UNDER VARIOUS EXPERIMENTAL CONDITIONS IN FED AND FASTED RATS[1*]

Animals	G-6-Pase activity per unit liver			
	Activity per 100 gm. wet weight		Activity per average cell	
	μg. P	Per cent†	μg. P×10^{-6}	Per cent†
Fed animals				
Normal	285±33 (100)†	100†	10.3±1.6 (100)	100†
Adrenalectomized	220±48 (77)†	77†	6.8§±0.9 (62)†	62†
Hypophysectomized	197§±40 (69)†	69†	6.4§±1.8 (59)†	59†
Fasted animals				
Normal	443±39 (100)‡	155†	10.9±1.8 (100)‡	106†
Adrenalectomized	334‖±44 (76)‡	118†	10.5±1.3 (89)‡	96†
Hypophysectomized	293‖±47 (66)‡	103†	6.8‖±1.9 (57)‡	62†
Hypophysectomized + sham operation	201‖±19 (45)‡	71†	6.3‖±0.9 (53)‡	58†
Hypophysectomized + partially hepatectomized	155‖±17 (35)‡	54†	5.2‖±06. (44)‡	48†

[1] See Table 1 for procedures and for statistical treatment of data.

* G-6-Pase activity is measured in μgm. of inorganic phosphate released per 15 minutes at 31° C. The enzymatic activity is expressed per wet weight and per average cell. Results are also expressed in per cent.

† Enzymatic activity expressed in per cent. The values of normal fed animals are taken as 100%.

‡ Enzymatic activities expressed in per cent. The values of normal fasted animals are taken as 100%.

§ Statistically significant difference as compared to values of normal fed animals ($P = <0.05$).

‖ Statistically significant difference as compared to values of normal fasted animals ($P = <0.05$).

sugar level in normal fasted animals averaged 77 mg. %. In the fasted adrenalectomized or hypophysectomized animals this blood sugar level was not maintained but decreased to about 40–50 mg.%.

The liver G-6-Pase activity of normal fasted animals was increased per unit wet weight, and it was maintained in the average cell. The total available liver G-6-Pase activity was increased in normal fasting animals (8).

In contrast to normal fasted animals the liver G-6-Pase activity in fasted adrenalectomized and hypophysectomized animals was decreased when expressed per wet weight, or per average cell. However, in the average cell

the decrease was statistically significant only in the hypophysectomized animals.

The total liver cell G-6-Pase activity per 100 gm. body weight was significantly decreased to 66–52% of that of the normal fasting values in both adrenalectomized and hypophysectomized rats. A parallelism between decreased blood sugar level and total liver G-6-Pase activity is shown in Table 3.

The Effect of Partial Hepatectomy and Sham Operation on Liver G-6-Pase

TABLE 3. COMPARISON OF RAT LIVER G-6-PASE ACTIVITY AND BLOOD SUGAR LEVELS UNDER VARIOUS EXPERIMENTAL CONDITIONS IN FED AND FASTED RATS[1]*

Animals	Absolute values		Per cent	
	G-6-Pase (μg. of P$\times 10^{-2}$)	Blood sugar (mg. per cent)	G-6-Pase	Blood sugar
Fed animals				
Normal	140.4 ± 21	115 ± 15	100	100
Adrenalectomized	87.1 ± 24†	85 ± 32	62	73
Hypophysectomized	79.0 ± 18†	128 ± 13	56	109
Fasted animals				
Normal	166.5 ± 9	77 ± 9	100	100
Adrenalectomized	109.5 ± 21‡	45 ± 16‡	66	58
Hypophysectomized and sham hepatectomized	85.8 ± 14‡	53 ± 18‡	52	69
	64.1 ± 10‡	21 ± 2‡	39	27
Hypophysectomized and partially hepapectomized	20.2 ± 3‡	18 ± 2‡	12	23

[1] See Table 1 for procedures and for statistical treatment of data.

* G-6-Pase activity is measured in micrograms of phosphorus liberated in 15 minutes at 31° C. The enzyme activity is expressed as total available liver G-6-Pase activity.

† Statistically significant difference as compared to values of normal fed animals ($P = <0.05$).

‡ Statistically significant difference as compared to values of normal fasted animals ($P = <0.05$).

Activity and Blood Sugar Levels in Fasted Hypophysectomized Animals. Groups of hypophysectomized animals were subjected to partial hepatectomy or sham operation and were put to fast and sacrificed 24 hours after the operation. Both sham operated and partially hepatectomized animals were in hypoglycemic coma when killed and showed very low blood sugar (Table 3) and G-6-Pase activity values (See Tables 2 and 3).

For the sake of demonstration of parallelism between blood sugar level and G-6-Pase activity all our available data were brought together in Figure 1. The effects of cortisone administration (8) and adrenalectomy in hypophysectomized rats were added to the data presented in this paper. This Figure shows that a parallelism between G-6-Pase activity and blood sugar level can only be demonstrated when the animals are fasted and G-6-Pase activity is expressed per 100 gm. body weight (total available activity).

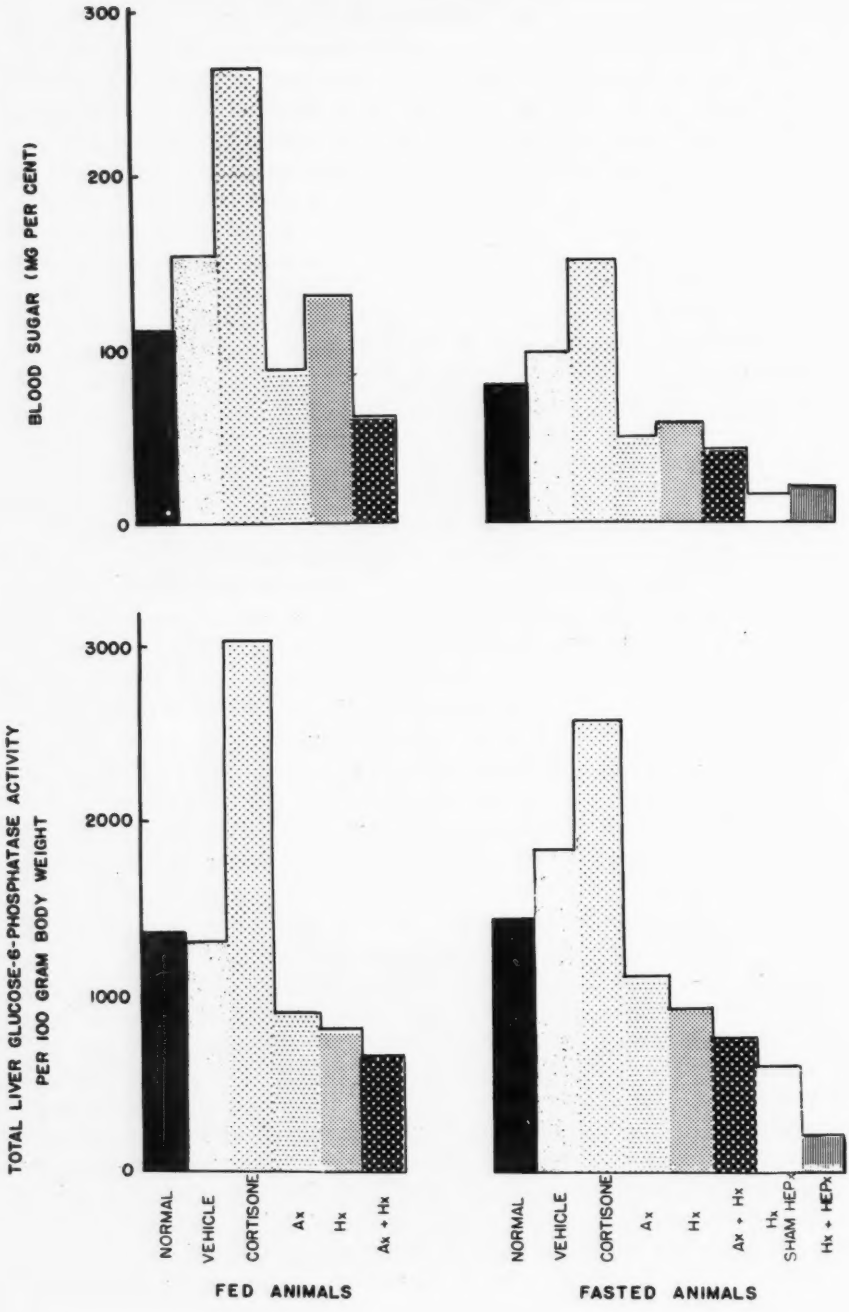
DISCUSSION

The Effect of Adrenalectomy and Hypophysectomy on Liver Constituents. Under these experimental conditions a significant decrease took place in liver weight, liver/body weight ratio and total liver nitrogen content in both fed and fasted animals. The change in liver/body weight ratio is of importance because G-6-Pase is chiefly concentrated in the liver (5) and the relation between the glucose production from the liver by the G-6-Pase reaction and the glucose uptake by the periphery is an important factor in determining the blood sugar level. The decrease in nitrogen content has to be taken into consideration because on the basis of a decreased protein content decreased enzymatic levels might be expected unless the enzyme is specifically maintained. This is of especial importance in the case of G-6-Pase which is in contrast with most liver enzymes is not decreased in fasting, but is maintained or increased in normal animals (2, 3, 4).

It may be noted that the cellularity (number of nuclei per unit weight of liver) in normal animals was higher than that reported by Einhorn *et al.* (21), and Price and Laird (22). These authors used the method of Price *et al.* (23) who made the nuclear counts after a 15 minute homogenization in sucrose, whereas we homogenized the tissue for a period of exactly 3 minutes in 0.85% saline. The importance of length of homogenization in determining nuclear counts was clearly demonstrated by Bass *et al.* (24).

The Effect of Adrenalectomy and Hypophysectomy on Blood Sugar Level and G-6-Pase Activity. In adrenalectomized or hypophysectomized animals when food was available *ad libitum* the blood sugar and G-6-Pase activity were in normal range. This agrees with findings of Ashmore *et al.* (15, 16). However, the picture is different when adrenalectomized or hypophysectomized animals are put to fast. These animals are unable to maintain normal fasting blood sugar levels. In normal fasting animals the total available G-6-Pase activity is increased, while other enzymes involved in G-6-P utilization are markedly decreased (16). On the other hand, in the absence of adrenal gland or hypophysis the fasting animal is unable to increase liver G-6-Pase activity to normal fasting levels.

In demonstrating decreased liver G-6-Pase activity after adrenalectomy and hypophysectomy the basis of expression of the enzymatic activity is of great importance. Due to the significant changes noted in liver weight, liver/body weight ratio and total liver nitrogen, the expression of enzymatic activities on the basis of unit wet weight, nitrogen or even per average cell could be misleading when a comparison is made between enzymatic activities and blood sugar levels. Therefore, the G-6-Pase activity was also expressed per 100 gm. body weight. Since G-6-Pase activity in the body is concentrated mainly in the liver, expressing total liver G-6-Pase activity per 100 gm. body weight is expected to give information about the "total available" G-6-Pase activity in the organism. In fasting the relation be-


BLOOD SUGAR (MG PER CENT)
300
200
100
0
TOTAL LIVER GLUCOSE-6-PHOSPHATASE ACTIVITY
PER 100 GRAM BODY WEIGHT
3000
2000
1000
0
NORMAL
VEHICLE
CORTISONE
Ax
Hx
Ax + Hx
FED ANIMALS
NORMAL
VEHICLE
CORTISONE
Ax
Hx
Ax + Hx
Hx
SHAM HEP
Hx + HEP
FASTED ANIMALS

tween hepatic glucose production and peripheral glucose consumption determines the glucose level; therefore, it was possible to demonstrate in fasting animals a parallelism between total available liver G-6-Pase activity and blood sugar level.

The decreased liver G-6-Pase activity in hypophysectomized animals may be explained, partially at least, on the basis of the role of the adrenal cortex in the regulation of liver G-6-Pase activity. That the presently described liver G-6-Pase activity decrease after hypophysectomy may be due to the absence of the adrenocorticotropic hormone of the hypophysis is strongly supported by the recent report of Shull *et al.* (17). These workers described that the hepatic G-6-Pase activity per gm. of tissue in mice bearing corticotropin (ACTH)-secreting tumors was markedly greater than in normal mice of the same strain.

The presented data also show that hypophysectomy causes a more marked decrease in liver G-6-Pase activity in both fed and fasted animals than adrenalectomy alone does. This finding indicates that the decreased hepatic G-6-Pase activity in hypophysectomized animals cannot be attributed solely to decreased or absent adrenocortical activity. This hypothesis is supported by the report of Glock and McLean (18) that thyroxin administration increased liver G-6-Pase activity. Therefore, it is possible that the effect of hypophysectomy on hepatic G-6-Pase activity is mediated through at least two endocrine organs, the adrenal gland and the thyroid gland.

Partial hepatectomy was performed in the present experimental series in order to remove surgically a large part of the G-6-Pase activity available for the animals. It was reported previously that when 66% of liver was removed from normal animals, the G-6-Pase activity was increased in the remaining lobe of the liver 1 day after operation (6). A similar increase was also found in animals 1 day after sham-hepatectomy. It was postulated that this increase might be due to the release of adrenocortical hormones as a result of the stress of anesthesia and surgery (6). In this connection it is of interest to note that when partial hepatectomy was done in hypophysectomized rats, these animals showed extremely low G-6-Pase activity in the remaining liver lobe 1 day after operation and the animals were in

FIG. 1. Comparison of blood sugar level and liver G-6-Pase activity in fed and fasted rats.—The blood sugar is expressed in mg. %. The enzyme activity is expressed as total available liver G-6-Pase activity. Normal = untreated animals. Cortisone = animals were given daily intramuscular injections of 25 mg. of cortisone acetate for 5 days and were sacrificed on the 6th day. Vehicle = animals injected the same way with the vehicle which contained only the suspending medium of cortisone, but no cortisone (8). Ax = adrenalectomized rats. Hx = hypophysectomized rats. Ax + Hx = adrenalectomized and hypophysectomized rats. Hx + SHAM HEPx = hypophysectomized rats which were subjected to sham-hepatectomy operation. Hx + HEPx = hypophysectomized animals which were subjected to partial hepatectomy.

hypoglycemic coma. The presented experiments also show that sham-operation which involves ether anesthesia, laparotomy and handling of the liver without removing tissue, also resulted in similarly lowered G-6-Pase activity and blood sugar levels.

The mechanism through which the adrenal gland and the hypophysis exert their effects on liver G-6-Pase is not clear. Two main possibilities may be considered here. 1) Hormones may have a direct effect on the enzyme. 2) Hormones may act through influencing gluconeogenesis which in turn could affect the G-6-P level in the cell. The studies of Ashmore and his colleagues (14) seem to support the second possibility. These workers have demonstrated that in the biological sequence after cortisone administration to adrenalectomized-diabetic rats the resulting increased gluconeogenesis preceded by several hours the increase in hepatic G-6-Pase. Thus, it seems unlikely that the increased glucose production is a direct result of the effect of the hormone on the enzyme in the case of the diabetic animal. On the other hand, it is possible that relations are different in the normal animal and the possibility of a direct effect on hormones on liver G-6-Pase should be further explored.

Statistical Significance and Biological Significance. The special case of liver G-6-Pase and blood sugar levels lends itself to the discussion on the statistical and biological significance of an enzymatic activity in relation to the product of the reaction. The comparison of blood sugar level and total available G-6-Pase activity demonstrates that a statistically significant per cent of the total available G-6-Pase activity may be lost without serious impairment to blood sugar levels in fasting animals. However, when the total available liver G-6-Pase activity falls under 50% of that of the normal fasting enzymatic level hypoglycemia and coma result (Table 3). These data might be taken as an indication that G-6-Pase in the liver is present in an excess of at least 50% to what is adequate to maintain the blood sugar level in the absence of food intake.

SUMMARY

The effect of adrenalectomy, hypophysectomy and partial hepatectomy on hepatic glucose-6-phosphatase activity and blood sugar level was investigated in fed and fasted rats. The enzymatic data were expressed per unit wet weight, per average cell and per 100 gm. body weight (total available activity). The enzymatic changes were interpreted in the light of alterations in liver weight, liver/body weight ratio, liver cellularity and nitrogen content.

In fed animals adrenalectomy or hypophysectomy caused a significant decrease in liver glucose-6-phosphatase activity on per cell basis. In fasted animals hypophysectomy, but not adrenalectomy, significantly decreased liver glucose-6-phosphatase activity. Partial hepatectomy or sham opera-

tion in hypophysectomized animals resulted in hypoglycemia and extremely low liver glucose-6-phosphatase values per unit wet weight and per average cell.

The total available glucose-6-phosphatase activity was significantly decreased in both fed and fasted animals under the various experimental conditions.

A parallelism between blood sugar and total available glucose-6-phosphatase activity can only be demonstrated in fasted animals under the experimental conditions reported here.

The significance of these findings was discussed.

ADDENDUM

While this manuscript was in preparation two abstracts were published by Menon and Gornall (19) and Harper (20) showing the effect of adrenalectomy and hypophysectomy on liver glucose-6-phosphatase activity per gm. wet weight. Their data are in essential agreement with those reported in the present paper.

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KETOGENIC AND ADIPOKINETIC ACTIVITIES OF PITUITARY HORMONES[1]

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IT HAS been known for many years that alkaline extracts of pituitary glands are capable of stimulating both ketosis and the development of fatty liver in various species. With the purification of pituitary hormones the evidence became increasingly suggestive that growth hormone was the factor which was responsible for the observed effects of pituitary extracts on fat metabolism. (For review of literature see references 1, 2, 3). From time to time irregular ketogenic responses to ACTH were reported and these were presumed to be due to the influence of adrenal cortical steroids (4, 5, 6, 7, 8, 9). However, doubt was cast on the latter interpretation by the demonstration that cortisone and cortisol inhibited fasting and other types of ketosis (10, 11, 12, 13). Moreover, when ACTH was administered to man by a slow intravenous drip which would be expected to cause maximal stimulation of adrenal cortical steroid secretion, fasting ketosis was suppressed (10).

In the meantime, Astwood, Raben, Rosenberg and their collaborators (1, 14, 15, 16) and ourselves (17, 18, 19) began to report effects of oxycel adsorbed ACTH on fat and carbohydrate metabolism which were identical with certain responses previously attributed to growth hormone. These included an adipokinetic response, ketosis, an increase in oxygen consumption, lowering of the R.Q. and hypoglycemia in intact and adrenalectomized animals. These findings explained some of the previously contradictory reports concerning the influences of ACTH and cortisone on ketosis and led to the conclusion that certain preparations of ACTH possessed metabolic activities which were not mediated by the adrenal cortex. They left unresolved the problem of whether these activities represented properties of the same molecule responsible for ascorbic acid depletion of the adrenal cortex or whether this factor was identical with or related to that associated with growth hormone and hence a contaminant of ACTH. The latter problem was further complicated by the demonstra-

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tion that thyroid stimulating hormone (TSH) also exhibited adipokinetic, ketogenic and hypoglycemic activities and the claim that these activities in growth hormone were in fact due to contamination with TSH (20, 21).

In the present report, an attempt is made to determine whether the ketogenic activity of ACTH, STH and TSH are due to a single or to multiple pituitary factors. The accidental observation that certain anesthetics potentiated fasting and other types of ketosis in the rat (22) offered an unexpected biological tool for exploring this problem when it was found that pentobarbital sodium anesthesia sensitized the rat to the ketogenic effect of ACTH but not to that of STH or TSH. The data suggest that each of the hormones independently possesses ketogenic activity.

METHODS AND MATERIALS

Male albino rats of the Vanderbilt strain and weighing 200–250 grams were used after a 24 hour fast. The ketogenic response was assayed as in a previous report (17) by estimating the increase in blood ketone bodies 3½ hours after the intraperitoneal injection of the test material. Anesthetized rats were kept under light pentobarbital sodium narcosis in a chick incubator at 33° C. The latter was necessary to prevent a fall in body temperature which was found to interfere with the development of ketosis. Unanesthetized rats were studied at room temperature and were restrained by wrapping loosely in a towel when blood samples were obtained from the tail. Blood ketone body levels were determined originally by a modification of the Greenberg and Lester method (23, 24) developed in this laboratory (25). In earlier studies a single heating was used but later the two-stage modification of Michael's *et al.* (26) was adopted. This yielded higher recoveries and accounts for the higher ketone levels in the later experiments. More recently a third modification of the method was introduced. It involved the use of salicylaldehyde (27) instead of 2,4 dinitrophenylhydrazine and carbon tetrachloride for the determination of acetone after digestion. Identical results were obtained by the two colorimetric methods but the salicylaldehyde technique was found to be much simpler.

Adipokinetic activity was estimated in female mice by the method of Rosenberg (15), the liver fat determination being done according to Handler (28). Food was removed from the mice's cage one hour before hormone injection. The animals were sacrificed 4 hours after ACTH and 7 hours after STH. Hormones were injected intraperitoneally.

The following hormone preparations were used:

Growth Hormone

1. Armour Laboratories. Bovine, Lots 22KRI, 3PKR-3 and R50109 (N.I.H.).
2. Dr. A. E. Wilhelmi, Bovine, Lots B168GH and B111-60.
3. Dr. C. H. Li—(Bovine).
4. Horner Co., Ltd., Montreal, Bovine, Lots C-35-39-J and 17A5110X and Porcine, Lot PR-1.

The bovine hormones were prepared for injection immediately prior to use by dissolving in water or saline and when necessary bringing to solution by adding 0.1N NaOH. In the latter case, the solutions were adjusted to a final pH of 8.0 with 0.1N HCl. PR-1, which was prepared from pig pituitaries by the technique of Raben and Westermeyer (29), was suspended in saline and brought more or less into solution by the addition of glacial acetic acid to pH 3.5.

Thyrotropin

1. Armour Laboratories—Lots M2004 (commercial) and IRW. Activity 1.5–2

U.S.P. units/mg. Lot IRW was estimated to contain 0.02 U.S.P. unit ACTH/mg. and negligible amounts of growth hormone. The hormones were dissolved in distilled water just before use.

2. Smith, Kline and French Laboratories—l-triiodothyronine. This material was dissolved in doubly distilled water brought to pH 9.0 with 0.1N NaOH.

Corticotropin

1. Wilson Laboratories—Oxycellulose-adsorbed ACTH—Lot 93359. 40 U.S.P. units/mg., 1 mg./ml. of phenol-water.
2. Dr. E. B. Astwood—Oxycellulose-adsorbed ACTH. 80 U.S.P. units/mg.
3. American Cyanamid Co.—Oxycellulose-adsorbed ACTH—Lot 10-OC 12.5 LAC S413-159. 80–100 U.S.P. units/mg.

The latter two preparations were dissolved in distilled water just prior to use.

RESULTS

Growth Hormone: Data on the ketogenic effects of a variety of growth hormone preparations in the rat are presented in Table 1 and are com-

TABLE 1. EFFECT OF GROWTH HORMONE ON RISE IN BLOOD KETONES IN 3½ HOURS

Hormone preparation	Dose in mg.	Mg.% increase as acetone	
		Nembutal	No nembutal
Control	—	+2.1±0.25 (15)	—
Armour—22KRI	0.300	*+5.0±0.72* (8)	—
Armour—22KRI	0.100	+2.9±1.17 (6)	—
Armour—3PKR-3	0.300	+5.4±2.10 (3)	—
Armour—3PKR-3	0.100	+4.7±0.89* (5)	—
Horner—C-35-39-J	0.300	*+3.5±0.25* (8)	—
Control	—	+2.0±0.30 (12)	—
Li	1.000	*+4.8±0.56* (12)	—
Li—0.1N NaOH—24 hrs.	1.000	*+4.0±0.42* (12)	—
Control	—	+2.7±0.31 (23)	−0.3±0.56 (8)
Wilhelmi—B168GH	1.000	*+6.3±0.56* (8)	*+5.9±1.02* (8)
Wilhelmi—B168GH—0.1N NaOH 24 hrs.	1.000	+4.1±0.59[a] (24)	—
Control	—	+3.1±0.57 (11)	—
Wilhelmi—B111-60	1.000	*+6.9±0.53* (4)	—
Wilhelmi—B111-60—0.1N NaOH—24 hrs.	1.000	*+6.0±0.68* (6)	—
Control	—	+3.6±0.31 (21)	−0.1±0.38 (12)
Horner—17A5110X	1.000	—	*+4.9±1.04* (5)
Horner—17A5110X	0.500	*+5.7±0.62* (10)	*+1.9±0.28* (10)
Horner—17A5110X	0.250	*+7.4±0.83* (10)	*+3.9±0.41* (8)
Horner—17A5110X	0.100	*+6.3±0.76* (9)	*+3.0±0.71* (10)
Horner—17A5110X	0.030	+3.4±0.62 (5)	+0.1±0.60 (4)
Horner—17A5110X—0.1N NaOH—24 hrs.	1.000	+4.2±0.17 (18)	+1.7±0.91 (18)
Horner—17A5110X—0.1N NaOH—24 hrs.	0.250	+3.9±0.85 (9)	+1.3±0.68 (10)
Control	—	+3.5±0.29 (16)	−0.1±0.38 (12)
Armour—R50109	0.200	—	*+3.2±0.56* (4)
Armour—R50109	0.100	*+6.0±0.69* (5)	*+2.3±0.83* (6)
Armour—R50109	0.050	+5.0±0.59[a] (8)	+0.4±0.48 (7)
Armour—R50109	0.025	—	+1.5±1.46 (4)

Numbers in parentheses represent No. of animals.
Values italicized—P < .01.
[a] P < .02.
* P < .05.

pared to the responses in corresponding controls which received an intraperitoneal injection of the inert solvent in each case. The lower control and experimental values in the first three groups are a reflection of the use of the older ketone method noted above. The most active preparations were the Armour 3PKR-3, R50109 and the Horner 17A5110-X which caused highly significant ($P < .01$) increases in blood ketone bodies at a dose of 0.100 mg. The Wilhelmi B168-GH and B111-60 and the Li bovine growth hormones were inactive at doses less than 1.00 mg. and the Horner C-35-39-J at doses below 0.300 mg. To conserve space these latter negative data are not recorded.

In previous reports (18, 30, 31) on the biological activities of porcine growth hormone (PR-1) prepared by the method of Raben and Westermeyer (29), it was found that leaving the hormone in contact with 0.1N NaOH for 16–20 hours at room temperature (25° C) had no effect on the ketogenic or adipokinetic activity but resulted in loss of growth activity by the tibial epiphysis assay in hypophysectomized rats, diabetogenic activity in the rat and hyperglycemic activity in man. In the present study, comparable studies with 0.1N NaOH treatment of growth hormone for 24 hours were carried out with several samples. As can be seen in Table 1, the Wilhelmi B-168GH and Horner 17A5110X preparations lost activity while the Wilhelmi B111-60 and Li hormones retained activity after 24 hours of alkali treatment. We have previously found that 16 hours treatment with 0.1N NaOH did not abolish the ketogenic activity of B-168GH (18). Russell (32) has also found that these same preparations still have nitrogen retaining activities in the nephrectomized rat after 16 hours but not after 24 hours of alkali treatment.

In the course of studies on the ketogenic activity of corticotropin, it was discovered that nembutal, phenobarbital and chloral hydrate anesthesia stimulated fasting ketosis in the rat and very greatly potentiated the ketogenic activity of corticotropin, as will be described further below. For this reason B168GH, 17A5110X and R50109 were retested in unanesthetized rats. As is apparent from Table 1, significant increments in blood ketone bodies occurred at approximately the same dosage of hormone irrespective of whether anesthesia was used or not. R50109 showed some activity at a dose of 0.050 mg. in anesthetized but not in unanesthetized rats which responded to 0.100 mg. In view of the relative lack of sensitivity of the assay, we do not consider this difference biologically significant. As will be discussed later, this behavior of growth hormone represents a significant difference from the response to ACTH which is increased many fold by anesthesia.

Measurements of liver fat in mice were carried out 7 hours after injection of the Wilhelmi B168GH and the Li growth hormones, before and after treatment with 0.1N NaOH at 25° C for 24 hours (Table 2). Both preparations exhibited significant adipokinetic activity at the doses used

and both showed partial loss of activity after alkali treatment. These results differ from those with the porcine growth hormone (Horner PR-1) and the porcine oxycel ACTH where alkali treatment seemed to increase adipokinetic activity. The data with PR-1 are presented in Table 2 for comparison. An actual increase in adipokinetic activity was observed after 16 hours exposure to 0.1N NaOH.

Thyrotropin: Two samples of Armour thyrotropin were investigated for

TABLE 2. ADIPOKINETIC ACTIVITY OF GROWTH HORMONE AND CORTICOTROPIN IN MICE

Hormone preparation	Dose in mg.	Liver fat, Gm.%
Control	—	8.2±0.39 (17)
Wilhelmi—STH—B-168GH	0.500	*11.5±0.53* (20)
Wilhelmi—STH—B-168GH—NaOH*	0.500	9.8±0.46[a] (16)
Control	—	7.9±0.28 (12)
Li—STH	0.750	*12.2±0.49* (8)
Li—STH—NaOH†	0.750	*10.5±0.51* (8)
Control	—	6.7±0.37 (14)
Horner—STH—Pr-1 pH 3.5	2.500	*8.8±0.49* (10)
Horner—STH—PR-1 NaOH†	2.500	*11.3±0.57* (10)
Control	—	5.7±0.29 (10)
Astwood Oxycel ACTH	0.100	*9.3±0.67* (9)
Astwood Oxycel ACTH NaOH*	0.100	*10.8±0.43* (10)
Astwood Oxycel ACTH NaOH‡	0.100	*9.5±0.51* (15)
Astwood Oxycel ACTH NaOH§	0.100	*7.4±0.27* (5)
Astwood Oxycel ACTH HAC‖	0.100	*12.3±0.51* (9)

* 0.1N NaOH for 24 hours at 25° C.
† 0.1N NaOH for 16 hours at 25° C.
‡ 0.1N NaOH for 10 min. at 100° C.
§ 0.1N NaOH for 30 min. at 100° C.
‖ HAC pH 3.5 at 100° for 30 min.
Mice sacrificed at 7 hours in the growth hormone study and 4 hours in the ACTH study.
Values italicized—$P < .01$.
[a] $P < .02$.

ketogenic activity. One was a commercial TSH (Armour) and the other (IRW) was more highly purified. With commercial TSH unequivocal activity was observed with a dose of 0.250 mg. in both anesthetized and unanesthetized rats and activity was destroyed by alkali incubation for 24 hours (Table 3). The more highly purified sample (IRW) showed significant activity in the anesthetized rat at a dose as low as 0.040 mg. and this activity was lost after incubation in 0.1N NaOH for 24 hours. The minimal active dose in the unanesthetized rat was 0.250 mg. The greater activity in the anesthetized rat is similar to that found with corticotropin, but the latter was stable in alkali.

Tri-iodothyronine in a dose of 0.005 mg. injected intraperitoneally had no influence on the ketosis (Table 3). This finding may be taken as some evidence against the concept that the ketogenic effect of TSH is mediated by thyroid stimulation, but studies on thyroidectomized rats are necessary to establish this point.

Corticotropin: Data have already been presented concerning the keto-

TABLE 3. EFFECT OF TSH ON RISE IN BLOOD KETONES IN 3½ HOURS

Hormone preparation	Dose in mg.	Mg.% increase as acetone	
		Nembutal	No nembutal
Control	—	+3.6±0.31 (21)	−0.1±0.38 (12)
Armour—M2004	0.500	*+8.7±0.94* (8)	*+5.2±0.58* (10)
Armour—M2004—0.1N NaOH—24 hours	0.500	+4.0±0.27 (7)	−1.2±0.58 (9)
Armour—M2004 " " " "	0.250	*+9.6±1.28* (4)	*+4.2±0.86* (4)
Armour—M2204 " " " "	0.100	+6.8±1.65 (9)	+2.0±1.58 (5)
Control	—	+3.5±0.29 (12)	−0.3±0.56 (8)
Armour—IRW	0.500	—	*+4.8±0.18* (4)
Armour—IRW	0.250	*+7.2±1.17* (4)	*+3.2±1.25* (8)
Armour—IRW	0.100	+5.1±0.57†(8)	+0.6±0.21 (8)
Armour—IRW	0.040	*+5.7±0.71* (7)	+0.8±0.22 (4)
Armour—IRW	0.020	+4.2±1.30 (8)	+0.7±0.11 (4)
Armour—IRW	0.010	+4.0±1.17 (4)	+0.7±0.21 (4)
Armour—IRW—0.1N NaOH—24 hours	0.250	+3.2±1.25 (8)	+0.2±0.13 (8)
Armour—IRW—0.1N NaOH—24 hours	0.040	+3.0±0.50 (7)	—
Triiodothyronine	0.005	+4.2±0.90 (7)	—

Numbers in parentheses represent No. of animals.
† P<.05.
Values italicized—P<.01.

genic, adipokinetic and hypoglycemic activities of untreated and alkali-treated oxycel corticotropin (17, 18) in anesthetized and unanesthetized rats. The present studies extend these observations to additional preparations and dosages of oxycel corticotropin. Table 4 records the results with preparations of oxycel corticotropin from three different laboratories. The Astwood oxycel ACTH which had been previously found to be active at 0.030 mg. but not at 0.003 mg. (17) was retested at the intermediate

TABLE 4. EFFECT OF OXYCEL CORTICOTROPIN ON RISE IN BLOOD KETONES IN 3½ HOURS

Hormone preparation	Dose in mg.	Mg.% increase as acetone	
		Nembutal	No nembutal
Control	—	+ 2.1±0.28 (15)	—
Astwood	0.030	+ *5.6±0.63* (9)	—
Astwood—0.1N NaOH—16 hours	0.030	+ *5.8±0.44* (23)	—
Astwood—0.1N NaOH—100°—10″	0.030	+ 2.5±0.34 (14)	—
Astwood—pH 3.5 HAC—100°—30″	0.030	+ *5.5±0.41* (15)	—
Control	—	+ 3.6±0.31 (21)	−0.3±0.56 (8)
Astwood	0.500	—	+0.5±0.24 (3)
Astwood	0.030	*+12.4±1.21* (19)	—
Astwood	0.010	+ *7.5±1.13* (8)	—
Wilson	0.134	*+14.0±1.99* (11)	—
Wilson—0.1N NaOH—24 hours	0.134	*+11.3±1.06*)6)	—
Wilson—0.1N NaOH—46 hours	0.134	+ *8.2±1.57* (7)	—
Wilson—0.1N NaOH—144 hours	0.134	+ 4.4±0.87 (8)	—
American Cyanamid	2.000	—	*+7.0±1.02* (8)
American Cyanamid—0.1N NaOH 24 hours	2.000	+ *7.7±1.59* (8)	*+2.5±0.44* (15)
" " " " " "	1.000	—	*+4.3±0.99* (8)
" " " " " "	0.500	—	*+3.1±0.94* (8)
" " " " " "	0.250	—	+0.9±0.67 (7)
" " " " " "	0.100	—	+1.7±0.80 (8)
" " " " " "	0.020	+ *8.5±1.27* (6)	—
" " " " " "	0.010	+ 3.8±0.79 (6)	—

Values italicized—P<.01.
Numbers in parentheses represent No. of animals.

dose of 0.010 mg. and found active in the anesthetized rat. In the unanesthetized rat it was inactive at 0.500 mg. and previously had been reported inactive at 1.00 and 2.00 mg. but active in a dose of 3.00 mg. (17). Since only enough material was available to test three rats at each dose, these latter results must be accepted with reservations. The American Cyanamid oxycel ACTH showed activity at 0.020 mg. but not at 0.010 mg. in anesthetized rats, while in the unanesthetized rats a significant response was obtained with a dose of 0.500 mg. The different preparations retained activity after exposure to 0.1N NaOH at 25° C from 16–46 hours, but activity was lost after 144 hours exposure and after 10 minutes in 0.1N NaOH at 100° C. The adrenal ascorbic acid depleting activity[2] of another sample of ACTH was not significantly reduced by 24 hours exposure to 0.1N NaOH but was abolished by heating to 100° C for 20 minutes (33). The Astwood oxycel ACTH heated to 100° for 30 minutes in acetic acid at pH 3.5 still showed ketogenic activity.

The fat mobilizing activity of the Astwood oxycel ACTH was also tested in mice (Table 2). In confirmation of the data of Rosenberg (15), it was found that significant fat mobilization could be demonstrated in 4 hours and that the dose of ACTH was relatively small compared to that required with STH. Adipokinetic activity was not destroyed either by 16 hours exposure to 0.1N NaOH at 25° C or 10 minutes at 100° C in acetic acid and actually was increased slightly after being heated to 100° C in 0.1N NaOH for 30 minutes at pH 3.5.

DISCUSSION

The present study confirms the fact that growth hormone, thyrotropin and corticotropin each have ketogenic activity in the rat and suggests that this response is not due to a single factor contaminating one or all of these hormones. Without knowledge concerning the chemical constitution and purity of the hormones used it is impossible to conclude this with certainty, but we consider the following observations pertinent. 1) The oxycel corticotropin samples tested were the most active ketogenic agents on a weight basis when tested in anesthetized rats. In terms of USP units however, the active doses were in the range of 0.8–1.6 units, which is far in excess of the reported ACTH contamination of the growth hormone and thyrotropin preparations used. Thus, thyrotropin IRW contained 0.02 USP unit ACTH/mg. and hence only 0.0008 U.S.P. unit was contained in the minimal active dose of IRW. The ketogenic action of growth hormone and TSH is thus probably not due to ACTH contamination. 2) Conversely, since the minimum effective doses of growth hormone and TSH required always exceeded those of ACTH in the anesthetized rat, it is hardly likely that the ketogenic action of ACTH is due to contamination with growth hormone or TSH. It could be argued that the major portion

[2] We are indebted to Dr. George Sayers for carrying out this assay.

of these hormone preparations is really inert and hence only a small amount of active contamination is necessary. This possibility cannot be ruled out until the minimal biologically effective molecules of growth hormone and TSH are identified and tested. However, since the ketogenic activity of corticotropin has been found in essentially pure corticotropin peptides (33), contamination seems improbable, unless the latter is exceedingly active, indeed. 3) The ketogenic activity of corticotropin always resisted mild alkali treatment whereas the other hormones often lost activity with this treatment. It might be noted that the corticotropin peptides behaved similarly to the oxycel corticotropins (33) and, as noted above, the adrenal ascorbic acid depleting activity of one of the above preparations was not modified by mild alkali treatment. On the other hand, Russell (32) has found that the growth response to and the amino acid retention activity of Wilhelmi growth hormone, Lot B-168GH, were lost with 24 hours alkali treatment. Our data indicate loss of both ketogenic and adipokinetic activities under the same conditions. The significance of these observations is limited by our ignorance concerning the precise chemical composition of the materials used by us and by the knowledge that active growth hormone samples may be prepared by quite diverse, methods and have rather different chemical properties. Thus, for example growth hormone prepared by the Wilhelmi method is quite stable at pH 9.0, while we have found that a Raben-Westermeyer growth hormone may lose growth and diabetogenic activity at this pH (30, 31), an observation also reported by Laso-Wasem and Graham (34). 4) The corticotropins exhibited 25–300 times greater ketogenic activity in anesthetized than in unanesthetized rats, whereas growth hormone exhibited equal activity and TSH showed an equivocally greater activity. It should be noted that anesthesia itself is a ketogenic stimulus since the blood ketone levels increase 2–3 mg.% in 3½ hours in the anesthetized rat while there is no change in the unanesthetized rat. This observation suggests that ACTH might have a different locus of action in ketone metabolism than does growth hormone or TSH and that nembutal anesthesia may act at the same or a related site. Phenobarbital and chloral hydrate have a similar action (22). This observation emphasizes another point of importance. The seemingly greater ketogenic potency of the corticotropins may not be real, since under physiological circumstances, i.e. in the unanesthetized rat, their activity is less than that of growth hormone and TSH. Adipokinetic activity was not measured in anesthetized mice and hence it is not known whether the same factors are at play in this response as in ketosis.

These data present relatively strong but indirect evidence that the ketogenic activity of ACTH is of a different origin from that of growth hormone and TSH. Studies in progress with purified corticotropin peptides strengthen this conclusion. The data reported here, however, are inadequate to determine whether the growth hormone and TSH have inde-

pendent ketogenic activities. Further investigation is necessary to affirm or deny the contention of Petersen and Lotspeich that TSH is responsible for the ketogenic activity of growth hormone (21). Finally, the data do not afford support for the concept that there is an adipokinetic-ketogenic factor independent of ACTH, STH and TSH but contaminating each of these hormones.

The demonstration that corticotropin has ketogenic activity which cannot be due to contamination with growth hormone takes on added significance when it is recalled that this and certain other metabolic activities are demonstrable even in the absence of the adrenal cortex and thus must be interpreted as extra-adrenal actions. ACTH has now been reported to have the following metabolic activities, many but not all of which have been searched for and found in adrenalectomized animals: Ketogenesis (1, 14, 17), adipokinesis (1, 15), R.Q. depression (1, 14), hypoglycemia in fasted animals (1, 17, 35), improved glucose tolerance (19), calorigenesis (1), cardiac glycogen deposition (1), and decreased urea formation from infused amino acid in nephrectomized rats (36). The fact that the same actions have always been attributed to growth hormone and that some are shared by TSH has important implications for the interpretations of pituitary hormone action. Suffice it to say that we consider it reasonable to suggest that some of these common metabolic activities may be anticipated in the sense that all the adenohypophyseal hormones are in final analysis growth and metabolic hormones for their respective target glands and therefore might be expected, under the proper experimental conditions, to exhibit certain activities which may be comparable to those of the systemic growth hormone, STH. Moreover, with large doses of a tropic hormone, one might anticipate that some of the activities which are considered specific for the target gland might be manifested in analogous reactions in other tissues. In these terms, the specificity of the tropic hormones may be due only in part to the structure of the hormone. A special affinity of the hormone for the gland or a greater sensitivity of the gland to the hormone coupled with the specialized organization of the target gland may in final analysis determine the seeming specificity of the tropic hormone for the target gland. Thus, for example, ACTH might be expected to stimulate the production of acetate from fat and its conversion to corticoids in the adrenal cortex; in the liver the end result of ACTH stimulation on acetate metabolism might be ketone body production. If this concept is correct, one might anticipate finding, under the proper experimental conditions, additional examples of actions of hormones on tissues other than on their target glands. Finally, it must be kept in mind too that a hormone obtained by chemical isolation may not necessarily have the precise chemical constitution of the secreted hormone and may thereby lose some of its specificity. Further discussion of the implication of these findings will be found elsewhere (33, 37).

SUMMARY

The ketogenic and adipokinetic activities of a variety of samples of growth hormone (STH), TSH and oxycellulose adsorbed ACTH, have been tested and compared in fasted rats.

On a weight basis the oxycel corticotropins were more active than STH and TSH when tested in anesthetized rats, but less so when tested in unanesthetized rats. The ketogenic activity of ACTH in rats under nembutal anesthesia was 25–300 times greater than in unanesthetized animals. STH and TSH had approximately equal ketogenic activity in anesthetized and unanesthetized rats. The significance of this phenomenon is discussed.

The ketogenic and adipokinetic activities of ACTH consistently resisted exposure to 0.1N NaOH for 16–24 hours at 25° C while the response of STH was irregular. Two samples of TSH lost ketogenic activity with alkali treatment. ACTH tended to lose metabolic activity with more prolonged exposure to alkali at 25° or on treatment at 100° C for 10–30 minutes. Where tested, native biological activity of the ACTH and STH correlated with ketogenic activity.

Analysis of the results in terms of the above parameters, the biological activity of each hormone preparation and the available information concerning the contamination of one hormone with another led to the conclusion that ACTH, STH and TSH each independently possess ketogenic activity, and this activity is not due to an independent ketogenic factor

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FURTHER STUDIES ON THE ADRENAL CORTEX AND CARBOHYDRATE METABOLISM

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WE HAVE already reported in a preliminary communication (39) that the subcutaneous injection of epinephrine into fasting adrenalectomized rats is followed by a greater fall in muscle glycogen than in intact rats and by practically no accumulation of liver glycogen in the four hour period following the injection of the hormone. Since the blood glucose and blood lactate changes are similar to those in intact rats, it follows that in the adrenalectomized animal a much larger proportion of the carbohydrate lost from the muscles has been disposed of over pathways that do not lead to liver glycogen accumulation.

Since the subcutaneous injection of epinephrine may be regarded as a test of the capacity of the organism to dispose of lactate, in this case derived from its own muscle glycogen, this paper reports not only more extended observations on the different response of intact and adrenalectomized rats to this hormone but also differences in the capacity of intact and adrenalectomized rats to form liver glycogen from other precursors.

METHODS

Male rats of the Wistar strain weighing 180–220 gm. were used throughout. They were all fasted for 24 hours prior to the experiment. Adrenalectomy was performed in one stage and the animals maintained for 7–14 days either by giving 1% NaCl as drinking fluid or by the injection of desoxycorticosterone acetate (DCA) (0.25 mg. /day).

Alloxan diabetes was produced by the intravenous injection of 4–5 mg. of alloxan per 100 gm. of body weight. The diabetic animals were kept in separate metabolism cages and the severity of the diabetes was determined from the urinary glucose excretion. Only those with high blood glucose levels at the end of a 24-hour fast were used.

All epinephrine injections, except where otherwise stated, were made into unanesthetized animals, the quantity given being 0.02 mg. of a freshly diluted solution of l-epinephrine hydrochloride (Adrin. Sharp and Dohme) which was injected subcutaneously.

At the end of the experimental periods all animals were deeply anesthetized by intraperitoneal injections of Nembutal. After a blood sample had been obtained from the vena cava or tail veins, one gastrocnemius muscle was rapidly removed, placed in a previously tared tube containing 2 ml. of 30% KOH. The tube was placed immediately into a boiling water bath until dissolution of the tissue was complete. Approximately 1 gm. of the right lobe of the liver was also removed and treated in the same manner as

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the muscle sample. The remainder of the liver was excised and weighed so that the total glycogen content of this organ could be determined.

The determination of muscle and liver glycogen was carried out by a modified Pfluger method and the glucose resulting from its hydrolysis, as well as blood glucose, was estimated by the Nelson method. Glycogen values are expressed as glucose. Blood lactic acid was determined by the method of Barker and Summerson.

In the experiments where various substances were infused, the animals were anesthetized with Nembutal, and a polyethylene cannula was inserted into a femoral vein. The substance was infused by means of a pump geared to deliver 2 ml. of fluid per hour. A total of 3 ml. was infused into each animal. The total time of infusion in all cases was 90 minutes.

Urine was collected during the infusion from a polyethylene catheter that was tied into the bladder. Urine samples were collected in small vials and frozen until analyzed.

The following substances were infused. (a) D-Glucose, A.R. anhydrous, (b) D-Fructose, twice recrystallized from acetone and water, (c) L-Lactic acid (Nutritional Biochemicals) partially neutralized with NaOH; the final pH of the solution administered was 4.0, (d) Glycerol, A.R., (e) L-Malic acid, which was also neutralized to pH 4.0 before injection. 1.67 mEq. of glucose and fructose and 3.34 mEq. of glycerol, lactic and malic acid dissolved in 3 ml. of water were given to each rat during the 90 minute infusion.

When hydrocortisone was administered it was given subcutaneously as a finely divided suspension in 0.9% NaCl, 5 hours prior to the sacrifice of the animals.

Calculation of total carbohydrate changes. The method introduced by Cori and Cori (7) for the calculation of the total muscle glycogen, free glucose and lactate in the body fluids has been used, with some modifications, for the calculation of the absolute changes in these constituents as a result of epinephrine injection.

These calculations depend on three assumptions: (a) that the muscles constitute 50% of the body weight of fasted animals; (b) that the free glucose and lactate is distributed in a volume equivalent to 25% of the body weight; (c) that the glycogen content of the gastrocnemius muscle may be used as the mean figure for the glycogen content of the whole skeletal musculature.

The experimental evidence justifying these assumptions is as follows: (a) Donaldson (9) found by actual measurement that in male rats weighing 220 gm. that the skeletal musculature constituted some 43% of the body weight. Since these were fed rats a figure of 50% is a reasonable estimate of the muscle mass in rats of this weight at the end of a 24-hour fast. (b) It has been shown in dogs and rabbits by means of radio-active glucose infusion, that the space occupied by diffusible glucose is about 25–30% of the body weight (35, 44). This agrees well with the values obtained by Sveinsson (36) by another method. In the fasted male rat Gey (13) has determined the true glucose content of a number of organs including the skeletal muscles and liver and related them to the true glucose content of the plasma. From his figures it is possible to calculate that the total free glucose content of the whole animal must be between 20–30% of the body weight. The use of the figure of 25% of the body weight, instead of 50%, as used by Cori and Cori would appear to give a more correct estimate of the total free glucose (and lactate) in the body. (c) The use of the glycogen content of a single gastrocnemius muscle as a measure of the average glycogen content of the whole of the skeletal musculature is the factor that would seem most open to question. There is, however, good reason to believe that such an estimate in the rat is not subject to such large and variable errors as in the case of the cat (11). Undoubtedly this is due to the greater uniformity in body size, nutritive state, etc. of the inbred laboratory reared animal. For example, Cori (8) observed in the rat that there was good agreement between an estimate of total muscle glycogen calculated from the content of the gastrocnemius and the total amount determined in the whole carcass. A similar relationship was also obtained by Evans (12),

while Long and Grant (25) found that the sum of the glycogen and lactic acid of the carcasses of both resting and exercised fasting rats is of the order of 230–240 mg. per 100 gm. of initial body weight. This would correspond to an initial gastrocnemius glycogen content of 460–480 mg.%, a value which is generally accepted as in the normal range for this muscle in the fasting animal.

RESULTS

THE EFFECT OF EPINEPHRINE ON THE CARBOHYDRATE METABOLISM OF FASTED NORMAL AND ADRENALECTOMIZED RATS

In Table 1 are shown the glycogen content in per cent of the wet weight of the liver and gastrocnemius muscles of intact and adrenalectomized

TABLE 1. THE EFFECT OF EPINEPHRINE ON THE LIVER AND MUSCLE GLYCOGEN, BLOOD GLUCOSE AND LACTIC ACID OF NORMAL AND ADRENALECTOMIZED FASTING RATS

	Number of rats	Liver glycogen	Muscle glycogen	Blood glucose	Blood lactic acid
Normal rats					
Controls	22	136± 16*	567±11	71± 2	22±1
1 hr. after Epinephrine	12	877±209	374±18	177±10	55±4
4 hrs. after Epinephrine	13	1694±120	385±22	115± 5	26±3
8 hrs. after Epinephrine	12	570± 23	424±32	76± 4	—
Adrenalectomized rats					
Controls—1% NaCl	18	70± 8	528±12	49± 4	18±1
1 hr. after Epinephrine—1% NaCl	12	36± 9	202±16	123±10	81±5
4 hrs. after Epinephrine—1% NaCl	12	156± 51	225±18	100± 4	19±1
8 hrs. after Epinephrine—1% NaCl	14	20± 5	237±16	43± 2	—
Controls—DCA	6	6± 3	518±12	62± 2	18±1
4 hrs. after Epinephrine—1% NaCl	6	201± 78	190±16	96±10	—

All values mg. per 100 gm. tissue.
* Standard error of the mean.

rats 1, 4 and 8 hours after the injection of the hormone, together with the blood glucose and lactic acid levels at these times.

It will be seen that 1 hour after the injection into intact rats there has occurred a 34% fall in muscle glycogen and a six fold increase in liver glycogen. This is accompanied by marked hyperglycemia and a large increase in blood lactic acid. Four hours after the injection, the loss of muscle glycogen is the same as at 1 hour but the liver glycogen is now increased some twelve fold over the initial level. The blood glucose is still elevated but the blood lactic acid has returned to the initial level. Eight hours later the liver glycogen has declined to one-third of its level at the fourth hour. The muscle glycogen still has not increased over the level reached the first hour after the injection.

In adrenalectomized rats the situation is very different. While the initial levels of liver and muscle glycogen and blood glucose are lower in

adrenalectomized animals, the fall in muscle glycogen, 1 hour after epinephrine, is twice as great (62%) as in intact animals. At this time there has been no significant increase, in fact a small decrease, in the concentration of glycogen in the liver. The levels of muscle and liver glycogen are essentially unchanged at the 4th and 8th hour after the injection, although there is some indication of a slight increase in liver glycogen at the 4th hour. Maintenance of the adrenalectomized rats by the daily injection of desoxycorticosterone acetate (DCA). instead of by the use of 1% NaCl as drinking fluid does not alter this atypical response to epinephrine (Table 1). Similar differences in the effect of epinephrine on the muscle

TABLE 2. CARBOHYDRATE BALANCES IN NORMAL AND ADRENALECTOMIZED RATS AT VARIOUS TIMES AFTER THE INJECTION OF EPINEPHRINE

	Liver glycogen	Muscle glycogen	Glucose in body fluids	Lactate in body fluids	Total	Net change
Normal rats						
Controls	5±0.6*	284± 5	18±0.6	6±1	313± 5	—
1 hr. after Epinephrine	31±7	187± 9	44±2.4	14±1	276±10	− 37
4 hrs. after Epinephrine	56±6	193±11	29±1.4	7±0.3	285±14	− 28
8 hrs. after Epinephrine	20±3	212±16	19±1	(6)†	257±15	− 56
Adrenalectomized rats						
Controls—1% NaCl	2±0.3	265± 7	12±0.5	5±0.2	284± 7	—
1 hr. after Epinephrine—1% NaCl	1±0.3	101± 8	31±2.5	21±1.2	154± 7	−130
4 hrs. after Epinephrine—1% NaCl	5±1	113± 9	25±1.6	5±0.2	148± 8	−136
8 hrs. after Epinephrine—1% NaCl	1±0.2	119± 8	11±0.2	(5)†	136± 7	−148
Controls—DCA	0.2±0.2	259± 6	15±0.2	5±0.3	279± 6	—
4 hrs. after Epinephrine—1% NaCl	7±0.3	95± 8	24±2.6	(5)†	131±10	−148

All values mg. per 100 gm. body weight.
* Standard error of the mean.
† Assumed to be the same as the initial value.

glycogen of intact and adrenalectomized rats have previously been reported by Collip, Thomson and Toby (5).

By use of the assumptions as to muscle mass, average muscle glycogen content and distribution volume of glucose and lactic acid in the body, it is possible to calculate the total changes in muscle and liver glycogen as well as in free glucose and lactic acid in the bodies of intact and adrenalectomized rats injected with epinephrine. These calculations are to be found in Table 2.

In the case of intact rats such calculations show the well known effect of epinephrine on the preformed carbohydrate stores of the body. Thus at the end of the first hour the loss of muscle glycogen is almost all accounted for by the increase in liver glycogen, blood glucose and lactic acid. There is, however, a small but significant amount of carbohydrate (37 mg. per 100 gm. of body weight) that is unaccounted for in these terms. At the fourth hour the difference in the total carbodyrate content of the controls and injected animals is no longer significant. Eight hours after the injection there is a significant loss of carbohydrate amounting to some 56 mg. per 100 gm. of rat.

In the adrenalectomized animals, by reason of the greater loss of muscle glycogen and its failure to appear as liver glycogen, there is at all periods examined a large proportion of the carbohydrate lost from the muscles that is unaccounted for as liver glycogen, glucose or lactate. This is about 100 mg. per 100 gm. of rat greater than is found in the intact rats at comparable periods. In fact at the 1- and 4-hour periods the carbohydrate unaccounted for in the adrenalectomized rat represents 78% and 90% respectively of the glycogen lost from the muscles under the action of epinephrine. It is also evident from the figures in Tables 1 and 2 that the disappearance of muscle carbohydrate occurs quite rapidly after the administration of epinephrine, in fact the major loss has occurred 1 hour after the injection. Presumably, in the absence of the adrenal cortical hormones a large portion of the carbohydrate, derived from the muscles, is utilized over pathways of metabolism that do not allow any significant portion to be accumulated as liver glycogen.

TABLE 3. EFFECT OF EPINEPHRINE ON THE MUSCLE AND LIVER GLYCOGEN LEVELS OF ANESTHETIZED INTACT AND ADRENALECTOMIZED RATS. MUSCLES REMOVED 1 HOUR APART

	No.	Muscle glycogen, mg.%			Liver glycogen, mg.%
		Initial	Final	Diffce.	
Normal	6	534±18	287±25	−247±8 (−46%)	1105±145
Adrex.	5	484±15	107±12	−377±8 (−78%)	62± 28

In another series of experiments the procedure was varied. Rats were anesthetized and one gastrocnemius muscle was removed along with a blood sample. Epinephrine was then injected and the animals kept under anesthesia for 1 hour at which time the second muscle along with a portion of the liver was removed for analysis. The results are in Table 3 and again show that in the adrenalectomized animals there is a significantly greater loss of muscle glycogen and a failure to deposit liver glycogen.

The failure of adrenalectomized animals to respond in a normal manner to epinephrine is not due to an inability of the hormone to release the muscle glycogen as lactic acid nor to a failure of the liver to increase the blood glucose from lactic acid. This is evident from the data on blood glucose and lactic acid given in Table 1 and is confirmed by a closer analysis of the time relationships of the response of these blood constituents after the injection (Fig. 1).

Except for a greater increase in the blood lactic acid 1 hour after injection into the adrenalectomized animals, and perhaps a somewhat sluggish response of the blood glucose, the curves are identical. In each case the peak of the blood lactic acid is reached at 1 hour and the initial levels are restored in 4 hours. The peak of the blood glucose does not occur until the

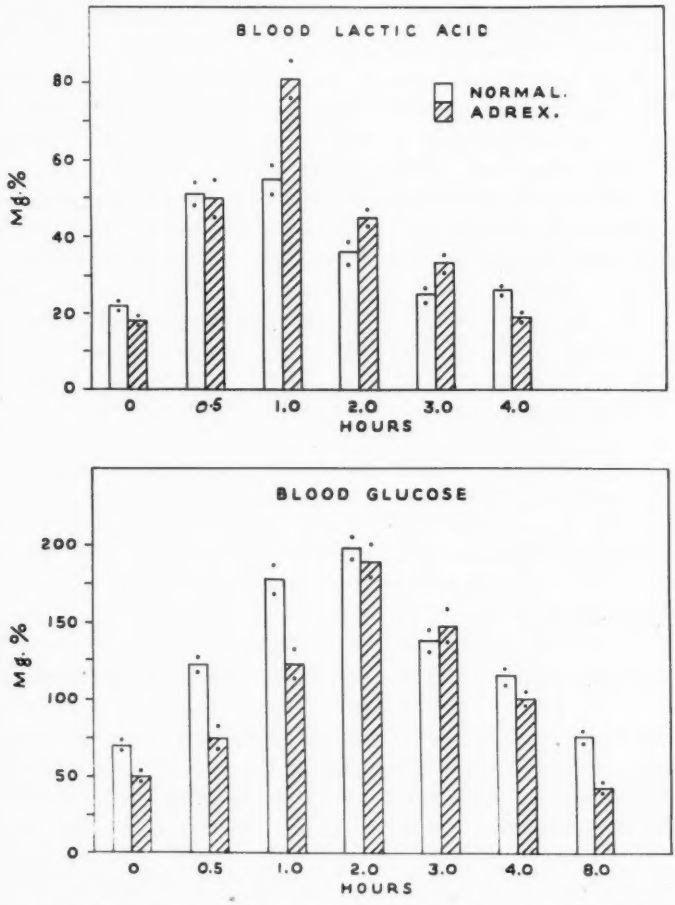

FIG. 1. The response of the blood glucose and lactic acid of fasted intact and adrenelectomized rats to the subcutaneous injection of epinephrine.

second hour and the initial levels are not reached again until somewhere between the fourth and eighth hour. There is no apparent difference, once the peak has been reached, in the rate of removal from the blood of lactic acid or glucose.

Effect of replacement therapy. It was noted above that the daily injection of DCA from the time of adrenalectomy did not restore the normal pattern of response of the carbohydrate metabolism to the injection of epinephrine. The effect of cortisone and adrenal cortical extract is shown in Table 4.

In all the experiments contained in Table 4 fasted adrenalectomized rats were used. They were either: (a) implanted at the time of operation

with cortisone pellets of about 9 mg. Some days later they were injected with epinephrine and sacrificed 4 hours later; (b) injected with 1 ml. an hour of aqueous cortical extract (ACE) during the 4-hour period of epinephrine action; (c) injected every 2 hours for 12 hours with 1 ml. of adrenal cortical extract prior to the injection of epinephrine, the injection of ACE being continued during the ensuing 4-hour period. These three groups, therefore, represent (a) the effect of a small, continuous but supportive amount of cortisone, (b) the effect of suddenly increased levels of adrenal steroids during the period of epinephrine action and (c) the effects

TABLE 4. EFFECT OF ADRENAL CORTICAL HORMONES ON THE RESPONSE OF THE ADRENALECTOMIZED RAT TO EPINEPHRINE

Group	Treatment	No.	Liver glycogen	Muscle glycogen	Glucose	Net
A	Cortisone pellet	2	2	303	15	
	Cortisone pellet+EPI.	4	32±5*	111±11	24±1	−153
B	ACE/ml./hr./4 hrs.	7	17±1	256±16	19±1	
	ACE/ml./hr./4 hrs.+EPI.	7	17±1	98± 8	35±2	−111
C	ACE 1 ml/2/hrs./16 hrs.	5	43±6	286±16	19±1	
	ACE 1 ml./2/hrs./16 hrs. +EPI.	7	92±4	187± 9	19±1	− 39

All values mg. per 100 gm. B.W.
* Standard error of the mean.

of excess of the cortical hormones both before and during the epinephrine period.

The effect of a small pellet of cortisone (Table 4A) is to partially restore the capacity of the liver to retain part of the muscle lactate as glycogen. It does not, however, prevent the excessive depletion of the muscle glycogen. In consequence there is still a large net loss of carbohydrate during the period.

The administration of cortical extract over a 4 hour period of epinephrine action increases the liver glycogen of the control group (Table 4B) and allows significant deposition of liver glycogen in the group given epinephrine. It does not, however, prevent the excessive loss of muscle glycogen and again there occurs a large net loss of carbohydrate.

On the other hand, rats pretreated with ACE for 12 hours prior to the injection of epinephrine, with continuance of the A.C.E. during the epinephrine period, show a pattern of carbohydrate response similar to that of intact rats (Table 4C). That is to say that in addition to a normal increment in liver glycogen there was also a considerably reduced loss of muscle glycogen (35% as compared to over 60% in Groups A and B). It should also be noted that in both Groups A and B that the increase in liver glycogen occurs in spite of the fact that in the control groups the level is already elevated by treatment with ACE alone.

The effect of epinephrine on eviscerated rats. In order to determine whether the greater loss of muscle glycogen in adrenalectomized rats given epinephrine was due to a greater sensitivity of the glycogenolytic mechanism of the muscles in the absence of the adrenal cortical hormones, the following experiment was performed.

One group of rats were adrenodemedullated, 3–4 weeks before use, in order to allow time for adequate regeneration of the cortical portion of the gland. This also ensures that the subsequent evisceration will not release large amounts of epinephrine and initiate the glycogenolytic process before the standard dose of epinephrine could be given. Another group of rats were totally adrenalectomized 1 week before use.

At the time of the experiment both groups were anesthetized and eviscerated. A subcutaneous injection of glucose, amounting to 20 mg. per 100 gm. of rat in the case of the adrenodemedullated animals and 40 mg. per 100 gm. in the case of the adrenalectomized animals was given. One gastrocnemius muscle and a blood sample was removed and the epinephrine injected subcutaneously. One hour later the second muscle and blood sample were taken.

It is apparent (Table 5) that the presence or absence of adrenal cortical tissue does not alter the rate of muscle glycogenolysis under the action of epinephrine. Consequently, the greater loss of carbohydrate observed in non-eviscerated adrenalectomized animals must be associated with the presence of one of the visceral organs.

TABLE 5. EFFECT OF EVISCERATION ON THE RESPONSE OF THE EVISCERATED RAT TO EPINEPHRINE

	No. of animals	Muscle glycogen, mg.%			Blood lactic acid, mg.%			Final blood glucose, mg.%
		Initial	Final	Decrease	Initial	Final	Increase	
Adreno-demed.	9	577±24	364±33	213±16	22±2	124± 7	102	62±10
Adrex.	8	568±21	329±26	239±16	18±2	130±10	112	68±12

The effect of epinephrine on alloxan diabetic rats

Since it is well known that adrenalectomized animals are unduly sensitive to insulin, there exists the possibility that the differences between them and intact animals is due to the unopposed action of insulin, secreted in response to the hyperglycemia produced by epinephrine. While there is, in fact, no evidence in these experiments of excessive insulin responses in the adrenalectomized animals, nevertheless, it was felt that this possibility should be definitely excluded.

Rats were rendered diabetic by the injection of alloxan. Animals were

selected that had exhibited severe glycosuria and had a high blood glucose at the end of a 24-hour fast. These were divided into four groups. (A) Control diabetic animals injected with saline and killed 4 hours later. (B) Diabetic animals who received epinephrine and were sacrificed 4 hours later. (C) A group of diabetic animals that were adrenalectomized 1 week before the period of fasting, injected with saline and killed 4 hours later. (D) A similar group of adrenalectomized-diabetic animals that were injected with epinephrine and sacrificed 4 hours later.

TABLE 6. EFFECT OF EPINEPHRINE ON THE CARBOHYDRATE METABOLISM OF ALLOXAN DIABETIC AND ADRENALECTOMIZED ALLOXAN DIABETIC RATS

Group	No.	Liver Glycogen	Muscle Glycogen	Glucose in body fluids	Urine glucose mg/4 hrs.	Total	Net change
A) Allox-diab.	11	108 +16*	250 ± 16	66 ± 7	8 ± 2	432 ± 19	—
B) Allox-diab. +EPI. (4 hrs.)	7	45 ± 8	89 ± 15	114 ± 11	113 ± 21	361 ± 19	− 71
C) Adrex allox.-diab.	5	1 ± 0.6	226 ± 11	14 ± 0.5	0	241 ± 12	—
D) Adrex allox.-diab. +EPI. (4 hrs.)	8	0.7 ± 0.4	39 ± 20	39 ± 8	0	79 ± 14	−162

All values mg. per 100 gm. body weight.
* Standard error of the mean.

The bladders of all animals were emptied by pressure prior to the injection of saline or epinephrine. Urine voided during the experimental period was collected and any urine remaining in the bladder at the time of sacrifice was also obtained.

The results in Table 6 are expressed as mg. of carbohydrate per 100 gm. of body weight. In the first place it will be noted that alloxan diabetic rats have, at the end of a 24-hour fast, much higher levels of liver glycogen than normal animals. This has been observed by several investigators (cf. 40). The average blood glucose of Group A at the end of the fast was 264 ± 27 mg. %, while the urinary glucose excretion during the 4 hour period averaged about 2–3 mg. per 100 gm. body weight an hour, values comparable to those observed by Scow (32) in fasting totally depancreatized rats. The total carbohydrate in the body of these fasting diabetic rats was therefore significantly greater than that of fasting normal rats. It should also be noted that the muscle glycogen content was slightly decreased.

The injection of epinephrine into such diabetic animals (Group B; Table 6) is followed by marked hyperglycemia, so that 4 hours later the blood glucose averaged 457 mg. % (Range 348–648). This was associated with heavy glycosuria indicating that a large proportion of the pre-existing stores of liver and muscle glycogen had been lost to the body. This is borne out by the observation that the liver glycogen falls in these animals under the action of epinephrine, in contrast to the increase that occurs in normal animals (Tables 1 and 2). There is also an exaggerated loss of muscle glycogen as compared to normal animals. This indicates, what is well known, that in normal animals part of the muscle glycogen lost under the

immediate action of epinephrine is restored to them by the action of the increased insulin secretion evoked by the hyperglycemia. In severely diabetic animals this part of the Cori cycle is largely inoperative, and a large proportion of the glucose, formed from lactate, that would normally replenish the muscle glycogen is now found in the body fluids and urine.

The important point, however, is that the net balance of carbohydrate is not unduly different from that of intact rats. The net loss of total carbohydrate (Table 6) as the result of epinephrine action in diabetic rats was

TABLE 7. EFFECT OF THE INFUSION OF VARIOUS SUBSTANCES ON CARBOHYDRATE METABOLISM OF INTACT, ADRENALECTOMIZED, AND ADRENALECTOMIZED RATS TREATED WITH HYDROCORTISONE

Substance infused	Amount Mg./100 g.m./B.W.	No.	Liver glycogen		Muscle glycogen, Mg.%	Blood sugar, mg.%		Urinary excretion, mg./100 gm./B.W.
			Mg.%	Mg./100 g.m./B.W		Initial	Final	
Normal								
None	—	4	117	4	478 ± 43	—	—	—
Glucose	122	6	1130	38	502 ± 11	73 ± 4	137 ± 7	0
Fructose	155	7	1900	71	530 ± 17	80 ± 6	153 ± 11	10 ± 3
Glycerol	119	6	1180	40	471 ± 14	69 ± 4	95 ± 4	—
Lactate	136	5	1420	44	528 ± 25	70 ± 2	87 ± 1	5 ± 2
Malate	142	5	570	18	479 ± 33	78 ± 3	98 ± 2	—
Adrex.								
None	—	11	27	1	457 ± 8	—	—	—
Glucose	130	6	500	16	489 ± 9	68 ± 6	198 ± 14	Trace
Fructose	124	6	810	25	477 ± 7	63 ± 4	151 ± 18	4 ± 0.8
Glycerol	130	7	280	9	406 ± 8	55 ± 3	85 ± 5	—
Lactate	119	8	128	3	444 ± 12	57 ± 3	84 ± 5	2 ± 0.5
Malate	149	6	31	1	539 ± 15	61 ± 3	101 ± 10	—
Adrex. + hydrocortisone								
None	—	4	1000	31	451 ± 10	—	—	—
Glucose	146	6	1980	66	454 ± 8	94 ± 5	259 ± 24	4 ± 4
Fructose	148	6	2970	108	467 ± 15	93 ± 2	179 ± 20	13 ± 1
Glycerol	145	5	1800	60	436 ± 18	85 ± 5	116 ± 8	—
Lactate	129	6	1730	54	484 ± 11	83 ± 1	114 ± 7	6 ± 2
Malate	151	6	1450	46	490 ± 19	96 ± 2	109 ± 12	—

71 mg. per 100 gm. as compared to a net loss of 28 mg. per 100 gm. in non-diabetic rats (Table 2). This difference is probably due to the difficulties of obtaining accurate measurements of urinary excretion of glucose during the period of action of epinephrine.

Groups C and D (Table 6) were adrenalectomized-alloxan diabetic rats given either saline or epinephrine. The control group exhibited the characteristic low blood glucose and liver glycogen and the absence of any glycosuria in the fasting state. A comparable group given epinephrine (Group D) showed, 4 hours later, a moderate degree of hyperglycemia, no accumulation of liver glycogen, a profound fall in muscle glycogen, and only traces of glucose in the urine. Consequently, there is a net loss of body carbohydrate of 162 mg. per 100 gm., an amount quite comparable to that unaccounted for in adrenalectomized rats treated in the same manner.

It is, therefore, apparent that loss of the adrenal cortical hormones affects the disposition of the carbohydrate mobilized by epinephrine in a

different manner than does the withdrawal of insulin secretion. In the case of the former, a large proportion of the carbohydrate is evidently disposed of over pathways that do not result in its appearance in the components determined in these experiments. Insulin lack, on the other hand, merely prevents the utilization of the glucose, formed from lactate, for muscle and perhaps liver glycogen synthesis, and in consequence large amounts of glucose appear in the body fluids and urine. Finally, these experiments emphasize that the effect of withdrawal of cortical hormones on carbohydrate metabolism in not dependent on the presence of insulin; a conclusion that is in keeping with the observation of Miller (26) that the administration of excess of adrenal cortical extract to adrenalectomized alloxan diabetic rats still results in a marked accumulation of liver glycogen.

Liver glycogen formation from various precursors in adrenalectomized rats

There have been several reports that after adrenalectomy both the intact animal and the isolated liver slice exhibit a diminished capacity to form liver glycogen from glucose, lactic, pyruvic acid and alanine (2, 3, 12, 16, 19, 24, 43). Conversely, the injection of adrenal cortical extracts into adrenalectomized animals or their addition to the medium containing liver slices, or even to the fluid perfusing isolated livers, has been shown to increase the liver glycogen content when lactate, glucose or alanine are used as glycogen precursors (6, 20, 28, 29, 33).

In view of the marked difference shown by adrenalectomized rats in the disposition of lactic acid released into the organism under the action of epinephrine, the ability of normal and adrenalectomized rats, and adrenalectomized rats treated with hydrocortisone, to dispose of several well known precursors of liver glycogen and blood glucose was reinvestigated. In these experiments, the details of which are described under Methods, the various meterials were infused at a constant rate into the animals over a relatively short period of time (1½ hours). The substances chosen for study in this manner were glucose, fructose, glycerol, lactate and malate. Table 7 shows the measured changes in liver and muscle glycogen as well as in the blood glucose as a consequence of the infusion of these substances into the three classes of animals employed. In Table 8 the amount and percentage of the infused material recovered as liver glycogen or sugar in the body fluids is calculated. It should be noted that with the amounts of the various substances infused and the relatively short period of infusion employed the changes in muscle glycogen have no significance. Consequently, the experiments test the relative capacity of the various groups of animals to form or retain a portion of the infused material as liver glycogen or, in the case of glycerol, lactate and/or malate, to also form glucose from them.

Glucose. At the amount and rate of glucose infused, normal rats retained some 28% as liver glycogen and added some 13% to the glucose in the body

fluids. After adrenalectomy only 12% of the material was accounted for as liver glycogen, but since the blood glucose rose to a greater degree some 25% was recovered as glucose in the body fluids. This apparent reduction in glucose tolerance after adrenalectomy in rats, as measured by blood glucose changes, has also been reported by Minibeck and Verzar (27) and Evans (12). The latter also noted the marked decrease in liver glycogen formation under these conditions. The administration of hydrocortisone (5 mg.) 3½ hours before the infusion elevated to normal the deposition of

TABLE 8. DISPOSITION OF VARIOUS SUBSTANCES BY INTACT AND ADRENALECTOMIZED RATS

Substance infused	Amount infused*	Deposited as liver glycogen*	in body fluids*	% of amount infused as:		% Accounted for
				Liver glycogen	Sugar in body fluids	
			Normal			
Glucose	122	34	16±2.5	27.9±2.1	13.1	41.0
Fructose	155	67	18±2.2	43.2±3.7	11.6	54.8
Glycerol	119	36	7±2.0	30.3±4.4	5.9	36.2
Lactate	136	40	4±0.8	29.4±2.1	2.9	32.3
Malate	142	14	5±1.0	9.9±2.4	3.5	12.4
			Adrex.			
Glucose	130	15	33±3.3	11.5±1.5	25.3	38.8
Fructose	124	24	22±4.8	19.4±1.9	17.7	37.3
Glycerol	130	8	8±1.5	6.2±1.7	6.2	12.4
Lactate	119	2	7±1.5	1.7±0.9	5.9	7.6
Malate	149	0	10±2.0	0	6.7	6.7
			Adrex.+Hydrocortisone			
Glucose	146	35	41±6.3	24.0±5.2	28.1	52.1
Fructose	148	77	22±2.2	52.0±3.8	14.9	66.9
Glycerol	145	29	8±1.0	20.0±7.0	5.5	25.5
Lactate	129	23	8±1.8	17.8±4.1	6.2	24.0
Malate	151	14	3±2.2	9.9±3.7	2.0	11.9

* Mg. per 100 gm. of body wt.

liver glycogen without significant effect on the quantity of glucose in the body fluids.

Fructose. Fructose is known to be a much better precursor of liver glycogen than glucose in intact rats. This is again illustrated in Tables 7 and 8 where the infusion of this sugar into intact animals resulted in the deposition of 43% of the amount given as liver glycogen in the period studied. Some 12% of the sugar was accounted for as reducing sugars in the body fluids (glucose+fructose). In the adrenalectomized animals the amount of reducing sugar rose slightly during the infusion but the proportion recovered as liver glycogen was reduced to less than half of that found in intact rats. Prior administration of hydrocortisone to the adrenalectomized

rats returned the liver glycogen deposition to normal amounts without any change in the amount of free sugar in the body.

Glycerol. In intact rats, this substance is as good a precursor of liver glycogen as glucose. It also brings about a moderate increase in the blood glucose level. After adrenalectomy, the same proportion of the amount infused is present as blood glucose, or in other words, the capacity of the liver to form blood glucose from glycerol is not impaired by adrenalectomy. However, there is a marked fall in amount of liver glycogen deposited. The prior administration of hydrocortisone, although not influencing the amount of glucose formation, returns the proportion of glycogen formed to amounts comparable to those observed in intact rats.

Lactic acid. The disposition of infused lactic in normal and adrenalectomized rats is entirely similar to that observed when an excess of lactate is liberated into the circulation by the administration of epinephrine (cf. above). That is to say, in intact animals a high proportion is recovered as liver glycogen while a smaller portion of the amount infused is accounted for by the increase in free glucose. After adrenalectomy, the quantity of infused lactate converted to free glucose is slightly increased while the proportion accounted for as liver glycogen undergoes a most striking decline. The prior administration of hydrocortisone to adrenalectomized animals, while it did not entirely restore the liver glycogen deposition to that found in intact rats, nonetheless brought about a substantial and unmistakable increase.

Malic acid. Even in intact animals malate is not a very effective precursor of liver glycogen, since only 10% of the infused material can be accounted for as the polysaccaride. It does, however, under the conditions used in these experiments, cause a moderate increase in the blood glucose. After adrenalectomy, liver glycogen deposition is not detectable at all, while glucose formation is unimpaired. Administration of hydrocortisone restored the proportion of liver glycogen deposition to normal levels.

The reduction from normal in liver glycogen formation in adrenalectomized rats per unit of precursor infused is 59% for glucose; 55% for fructose; 80% for glycerol; 94% for lactate and 100% for malate. The capacity to form free glucose in the body fluids from glycerol, lactate or malate is unchanged while in the case of glucose a relative inability to dispose of this sugar appears to be present after adrenalectomy.

The urinary excretion of glucose, fructose and lactate was measured in all three groups of animals studied. As Table 7 shows the differences between intact and adrenalectomized animals could not be accounted for by a more rapid and extensive excretion of these compounds. In the case of lactate, the blood levels, before and at the end of the infusion, were measured. In intact animals the increase in blood lactate was 50 ± 8 mg. %; in adrenalectomized animals it was 48 ± 4 mg. %; and in adrenalectomized animals treated with hydrocortisone it was 57 ± 4 mg. %. Evidently there

is no difference in the ability of these various groups of animals to remove infused lactate from the blood. This observation is in agreement with that found in the case of the blood lactate clearance after epinephrine injection (cf. Figure 1.).

DISCUSSION

These studies indicate that the adrenal cortical hormones have a marked effect on the disposition of carbohydrate. The change in the pattern of metabolism of glucose, fructose, glycerol, lactate, and malate following adrenalectomy suggests that there is a particular pathway or pathways of metabolism that operate to greater than normal degree in the absence of the cortical hormones, and conversely are diminished when these hormones are present in excess.

The experiments reported here while they do not permit any precise localization of the site of action of these hormones do allow certain deductions of the general nature of their effect. In the first place the differences in the response to epinephrine injection are not due to any change in the glycogenolytic activity of this hormone on muscles; nor are they due to any gross failure of the liver to form glucose from the lactate reaching it from the muscles. Rather, the atypical "Cori cycle" in adrenalectomized rats is characterized by a failure to deposit any significant amount of the glucose formed from lactate either as muscle or liver glycogen, a failure that is restored by the administration of the 11–17 hydroxy adrenal steroids. Consequently, since neither lactate or glucose accumulate in the body fluids to an excess degree, it must follow that they have been utilized to a far greater extent than normal over other pathways of metabolism. Furthermore, the presence or absence of insulin does not alter the consequences of either adrenal cortical insufficiency or excess. It may, therefore, be concluded that although both insulin and adrenal cortical insufficiency have certain overall affects on carbohydrate metabolism, such as hypoglycemia, in common, the mechanisms operating to produce them are not interdependent nor necessarily related.

This atypical disposition of the lactate produced by the action of epinephrine is also seen when other materials known for their capacity to increase liver glycogen in normal rats are infused into animals deprived of their adrenals. In the case of the two sugars, glucose and fructose, the proportion recovered as liver glycogen undergoes a large decrease while the decline in liver glycogen formation from glycerol, lactate, and malate is even greater. In the case of all these compounds there is again no evidence that this difference is due to a failure to clear them from the blood at an adequate rate or to excessive urinary loss. Nor in the case of the last three is it due to a failure to form glucose, at least as judged by the normal increase in blood glucose after their infusion. Since a normal quantity of liver glycogen deposition occurs if the adrenalectomized rats are pretreated with hydro-

cortisone it must be concluded that adrenalectomy results in an excessive utilization of either carbohydrates or their precursors over some other pathway.

There is also evidence from these experiments to suggest that the liver is the organ upon which these effects of the adrenal cortical hormones are first exerted. This is indicated by the absence of any notable differences in the lactate metabolism of eviscerated and adrenalectomized eviscerated animals. While it might be argued that this would be anticipated if it was the glucose utilization of the muscles alone that was affected by adrenalectomy, it should be noted that Russell (31) was unable to observe any notable differences in the glucose tolerance of adrenalectomized eviscerated rats maintained on DCA when compared to normal eviscerated animals, although she found that adrenal cortical extract reduced the glucose tolerance of saline-maintained adrenalectomized and of hypophysectomized animals.

Wick and Drury (45) and Cohn *et al.* (4) reported no effect of adrenalectomy or of adrenal steroids on the glucose metabolism of eviscerated rabbits and dogs. Ingle *et al.* (18) found that adrenal cortical extract had a small, delayed effect on the glucose tolerance of eviscerated rats. Selye and Dosne (34) have reported that adrenal cortical extract does not alter the rate of fall of blood glucose in such animals. Furthermore Levine and his colleagues (22) found that the extreme insulin sensitivity of adrenalectomized rats was abolished after evisceration. There is also no indication that adrenalectomy alters the glucose uptake of the isolated rat diaphragm (4, 19, 21, 23, 38). It should be remembered that the surgical procedure is drastic and that important metabolic activity of the carcass may be suppressed to a minimum by evisceration. The lack of a consistent immediate or striking effect of adrenalectomy on the carbohydrate metabolism of the muscles is in sharp contrast to the prompt effect of these hormones on the carbohydrate metabolism of intact animals and on that of the perfused liver or liver slice.

The alternate pathways of metabolism, the extent of whose operation is subject to control by the adrenal cortical hormones is not known. It has been suggested by some and denied by others that lipogenesis in the liver is such a pathway. In the experiments reported in this paper, the change in the fatty acid content of the liver 4 hours after giving epinephrine to adrenalectomized rats was determined. No significant increase was found over that of untreated animals, yet in this period about 100 mg. of carbohydrate was unaccounted as glycogen or glucose.

The most obvious pathway by which the temporary excess of carbohydrate or its precursors might be utilized is by increased oxidation to CO_2. Winternitz and Kline (41) have reported that adrenalectomized rats do have a higher respiratory quotient than normal animals after epinephrine

injection. Russell (30) did not observe any higher RQ in glucose fed adrenalectomized animals although Thorn *et al.* (37) found such an increase in individuals with Addison's disease.

One other possibility is that these hormones control the proportion of glucose that is metabolized through the phospho-gluconate shunt. There is some indirect evidence that they have such an effect. Glock and McLean (14) found that the content of glucose 6-phosphate and 6-phospho-gluconate dehydrogenases of the liver was decreased in alloxan diabetic rats, a condition in which there is a relative or even an absolute increase in adrenal cortical activity. A hormonal effect exerted on this pathway of carbohydrate metabolism would have far reaching consequences on the overall metabolism of both carbohydrate and protein since not only would carbohydrate utilization be affected but what is more important the production of pentose phosphates. Since the latter are essential components of nucleotides and nucleic acids, widespread alterations in protein metabolism could be expected to follow variations in the level of cortical hormones in the body. Furthermore, such an effect might explain the well known relationship between the carbohydrate and protein metabolism that is such a characteristic feature of either a lack or excess of these hormones. There is already some evidence that the nucleotide and nucleic acid metabolism is influenced by cortical hormones. Glock and McLean (15) observed that the total diphosphopyridine nucleotide content of the liver was reduced in alloxan diabetic rats, while several papers have reported that excess of cortisone reduced the ribose nucleic acid content of liver and other tissues (1, 10, 17, 42).

It may well be that the association of a depression of carbohydrate utilization and negative nitrogen balance which is such a characteristic feature of excess of cortical hormones, as well as a consequence of injury of various kinds, has its origin in these alterations in nucleic acid and nucleotide metabolism; alterations that are in part at least determined by the changing levels of these hormones in the body.

SUMMARY

The subcutaneous injection of epinephrine into fasted adrenalectomized rats is followed by a decline in muscle glycogen almost twice as large as in intact animals. Furthermore, in contrast to intact animals there is practically no accumulation of liver glycogen. Injection of adrenal cortical hormones restores the normal pattern of response.

This difference in response is not due (a) to a failure to remove lactate from the blood or (b) to an inability to form glucose from lactate.

In consequence, in adrenalectomized rats there is a large portion of the muscle glycogen, mobilized by epinephrine, that is not accounted for as liver glycogen or increased glucose in the body fluids.

Eviscerated adrenalectomized rats have a normal response to epinephrine. Evidently, a changed pattern of metabolism in the liver is responsible for the effects observed.

The increased, and unaccounted for, disappearance of muscle glycogen is also found in adrenalectomized alloxan-diabetic rats.

The infusion of glucose, fructose, glycerol, lactate and malate into fasted adrenalectomized rats is followed by much less glycogen deposition than in intact animals. This is restored by prior treatment with hydrocortisone. The increase in blood glucose after glycerol, lactate or malate infusion is not changed by adrenalectomy.

It is suggested (a) that the liver is the site of the immediate effect of the adrenal cortical hormones on carbohydrate metabolism; (b) that these hormones may regulate both in the liver and other organs the extent to which glucose is utilized over alternate pathways of metabolism. Possible pathways that may be affected are discussed.

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STRUCTURE AND FUNCTION OF INTRAOCULAR HYPOPHYSEAL GRAFTS IN THE HYPOPHYSECTOMIZED MALE RAT[1,2]

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IT IS becoming increasingly probable that the classical "feed-back" or "servo" theory of target gland-pituitary interplay does not adequately describe the actual glandular interrelations. For example, studies of various metabolic stimulants and pyrogenic agents show that the level of plasma protein bound iodine (PBI) does not necessarily regulate output of thyroid-stimulating hormone (TSH) by the pituitary (1). Such output can be suppressed even in rats synthesizing *no* thyroid hormone so long as a normal or high metabolic rate is maintained (1). The results of these studies suggest that not the actual level of circulating thyroid hormone, but one (or more) of its metabolic effects acting directly or indirectly through the central nervous system is (or are) the crucial element(s) in the regulation of pituitary output of TSH. If the determining substance(s) act directly on the pituitary beta cells (source of TSH), the actual anatomical location of these cells, assuming adequate vascularization, should not be of great importance. Hence, it was of interest to investigate how well such cells would develop and function when removed from their normal anatomical relation to the central nervous system. Similar considerations apply to the mechanisms regulating the secretion of ACTH and the gonadotropins (2). The present report deals with observations on the cytology and function of rat fetal pituitary tissue grafted into the anterior chamber of the eye of hypophysectomized male rats.

MATERIALS AND METHODS

Pituitary tissue used for implantation was obtained from rat fetuses, removed from the uterus on the 21st day of gestation (3). Male rats (Sprague-Dawley) were hypophysectomized by the parapharyngeal route, and seven days after the operation those in which ablation of the pituitary was deemed complete were selected as implant recipients.

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At transplantation the fetuses were removed from the uterus of the lightly (ether) anesthetized mother; the fetal pituitary removed with the aid of a dissecting microscope and drawn into a micropipette. A slit was made in the superior segment of the cornea of recipient rats with a no. 15 Bard–Parker scalpel and one fetal pituitary was placed in each eye.

Intact and hypophysectomized rats of the same age and maintained in a manner identical to the experimental groups served as controls.

The thyroid-serum iodide concentration (T/S) ratio was determined by a method described elsewhere (4). Adrenal cholesterol depletion studies were carried out by removal of the left adrenal under ether anesthesia, followed by removal of the right gland 4 hours later; adrenal cholesterol was determined by the method of Knobil *et al.* (5).

One lobe of the thyroid was used for determination of radioactivity; the other was fixed in formalin. Each lobe was weighed separately. The testes, seminal vesicles and prostate were weighed and fixed in formalin; the eyes were enucleated and fixed in sublimate-formol for 24 hours.

The thyroids, testes and prostate glands were sectioned at 5 μ and stained with hematoxylin and eosin. The brain was fixed *in situ* and sectioned serially at 10 μ following decalcification of the bony case. Alternate sections were stained with hematoxylin and eosin; sections through the region of the hypophysis were stained by methods (6, 7, 8) used for the implanted pituitaries (below).

The eyes were embedded in paraffin and serially sectioned at 3 or 4 μ. Each strip of 15–20 sections was divided into thirds. The first third was stained by a modification (6) of Martins' (7) stain: the second with periodic acid-Schiff, and the third with Gomori's (8) aldehydefuchsin (A–F).

RESULTS

Rats suspected of having residual intracranial hypophyseal tissue either on the basis of postoperative growth and development or from examination of serial sections of the brain were not included in this report. Examination of such serial sections revealed that almost all of these hypophysectomized rats contained, in the region of the stalk or tuber cinereum, cells or groups of cells whose precise identity could not be ascertained by morphological or tinctorial criteria (Fig. 1.).

Within one or two weeks after transplantation one could readily determine if the implant was viable merely by macroscopic examination of the eye. A striking feature was the rich vascular supply of the implants which appeared to derive from the uveal system. Whereas differences in behavior were not noted between the rats bearing implants and the hypophysectomized controls, there was a slight but significantly greater growth rate ($P < .05$) in the implant-bearing group (Table 1). There was no replacement of the immature pelt in the hypophysectomized controls in contrast to a clear transition to the adult type in about one-third of the implant-bearing rats.

Testicular descent and growth was noted in four of ten rats bearing ocular grafts. Initial testicular descent occurred at 1,4 or 6 months after operation; oddly enough the testes descended in all four rats when they attained a body weight of 145–150 gm. These rats were placed with virgin females and in all cases these were impregnated, yielding normal litters.

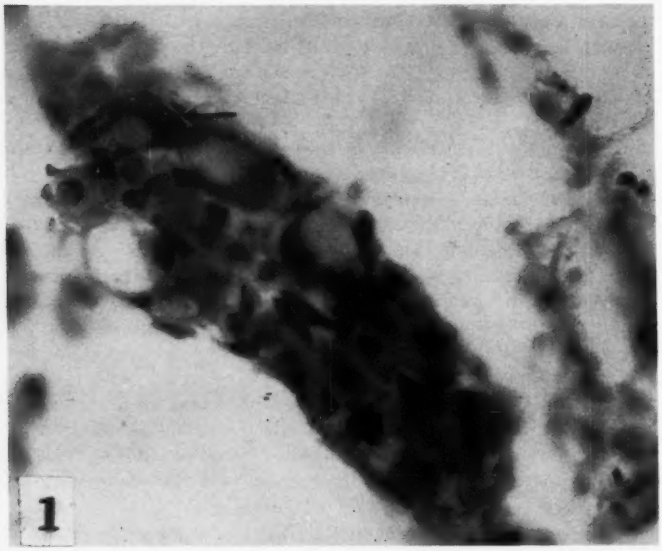

FIG. 1. Arrow points to a cell of the hypophyseal type with structure similar to negative image of Golgi apparatus. This cell had aniline-blue PAS-positive granules. Martins; ×800.

No discernible effect on the growth rate, pelt, appearance, or activity of the implant-bearing rats could be noted following the daily administration of 1.4 μg. of dl-thyroxine. This was administered by the subcutaneous route to 4 of the implanted rats for eight weeks

Intraocular Implants

It was evident from the amount of intraocular hypophyseal tissue found in most animals at autopsy that 1) growth of the graft had occurred and 2) such tissue can remain viable for periods of up to eight months. In all but three of the implanted rats, tissue representative of all three (anterior,

TABLE 1. EFFECT OF PITUITARY IMPLANTS ON GROWTH, THYROID, AND ADRENALS OF HYPOPHYSECTOMIZED RATS

Group	Final body weight, gm.	Adrenal gland			Thyroid gland		
		Absolute weight, mg.	Relative weight, mg./100 gm.	Cholesterol depletion, % change	Absolute weight, mg.	Relative weight, mg./100 gm.	T/S ratio
I. Transplant	161± 8[0]	14.96±0.63[1]	9.49+0.71[2]	− 4.3+2.8[3]	11.63±0.68[4]	7.34±0.50[5]	7.59±1.32[6]
II. Hypox	144± 4	10.99±0.50	7.64±0.31	+13.1±2.6	9.81±0.35	6.89±0.33	2.28±0.15
III. Intact Controls	460± 10	47.5 ±3.2	10.30±0.90	−29.2±3.7	18.21±1.8	3.98±0.24	12.9 ±2.8

P Values (I vs. II):
[0] $P<.05$
[1] $P<.01$
[2] $P<.05$
[3] $P<.01$
[4] $P<.02$
[5] $P<.05$
[6] $P<.01$
Values are Mean ± standard deviation.

posterior, and intermediate) lobes was found (Fig. 2). The residual cleft was often quite well defined. In two of the implants anteroir lobe cysts of the type occasionally found in normal pituitaries (*in situ*) were present; one was lined with squamous epithelium, the other with ciliated columnar epithelium with interspersed goblet cells. Although India ink injection studies were not made, it was clear that the glands were well vascularized.

The anterior lobe of the implants was largely composed of chromophobic cells with vesicular nuclei and prominent nucleoli (Fig. 3). The alpha cells (acidophils) were sparse and not densely granulated. They

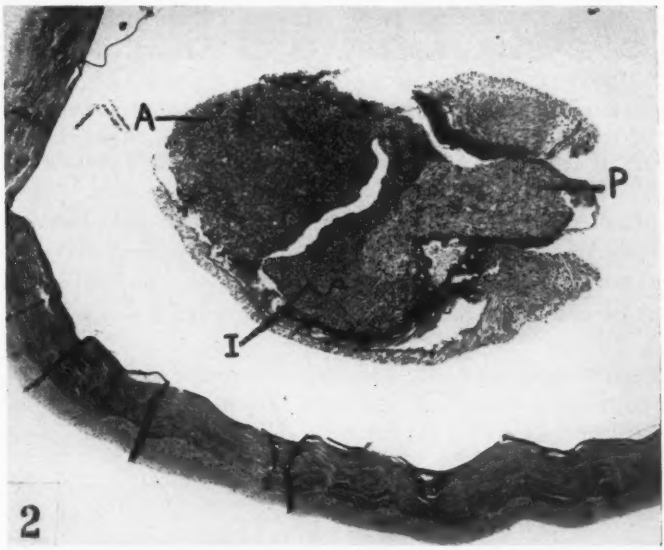

FIG. 2. Implant of pituitary in anterior chamber of eye. Note anterior lobe (A), intermediate lobe (I) and posterior lobe (P). There is a well-marked "residual cleft" between the anterior and intermediate lobes. Martins; ×60.

appeared smaller than those in sellar tissue and constituted less than 10% (estimated) of the total cell population.

The "basophil" cells stained quite faintly with aniline blue, contained fine granules and a small "negative image" of the Golgi apparatus. These were in even fewer numbers than the alpha cells. None exhibited the morphological characteristics of active secretion (i.e. large Golgi apparatus, vacuole formation, cellular and nuclear hypertrophy). Angular basophilic cells, filled with granules strongly positive to the periodic acid-Schiff reaction and to Gomori's aldehyde-fuchsin, were scattered throughout the implants. These cells (Fig. 4) were cytologically quite similar to if not identical with the TSH-secreting beta cells of sellar anterior lobe tissue. Aldehyde-fuchsin negative, PAS-positive basophils resembling delta cells were rare, even in the rats with testicular maturation.

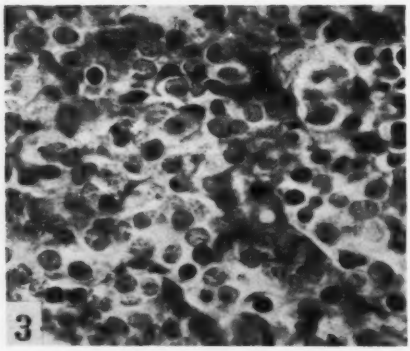

FIG. 3. Higher magnification of field from anterior lobe portion of an intraocular implant. Red blood cells appear black. Martins; ×850.

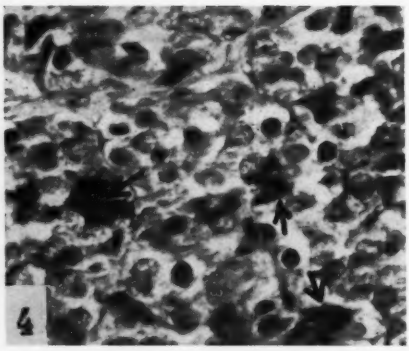

FIG. 4. Portion of anterior lobe of intraocular implant. Arrows point to beta cells, containing deep (purple) staining granulation. Gomori aldehyde-fuchsin; ×710.

The intermediate lobe was composed of regular pale basophilic cells (Fig. 5) comparable to those of the normal (sellar) intermediate lobe. Their granules stained not at all or much less intensely with either aldehyde-fuchsin or periodic acid-Schiff than did the normal intermediate lobe cell granules.

The posterior lobe was composed of numerous cells with vesicular nuclei, embedded in a rich fibrillar stroma (Fig. 6). There was no aldehyde-fuchsin positive material in this tissue.

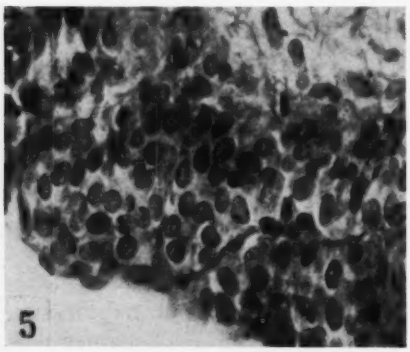

FIG. 5. Higher magnification of the residual cleft and intermediate lobe. The cells contain round to oval vesicular nuclei and the granulation stains faintly to deeply with the periodic acid-Schiff technique and with the Gomori aldehyde-fuchsin. A portion of the posterior lobe may be seen at the upper right corner. Martins; ×710.

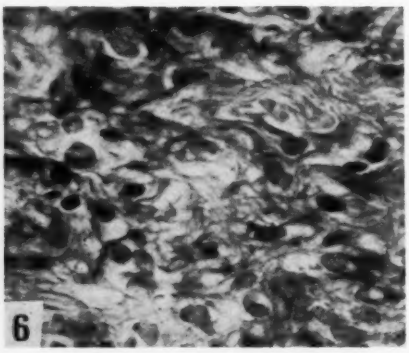

FIG. 6. Higher magnification of the posterior lobe. Note the abundance of fibrils which stain very intensely with aniline blue, and the scattered round cells of the glial type. Martins; ×710.

TABLE 2. CONDITION OF GONADS AND ACCESSORY TISSUE IN IMPLANTED RATS

Group	Rat	Body weight, gm.	Testis weight, mg.	Seminal ves. wgt., mg.	Prostate gland wgt., mg.	Histology of testis
Intact controls	50	492	3560	830	741	Normal
	51	474	3398	790	640	Normal
	53	490	3742	750	778	Normal
	54	443	3460	700	762	Normal
	55	423	3160	793	550	Normal
	57	440	3350	653	604	Normal
	avg.	460	3795	753	679	
Hypox	37	136	297	16	8	Atrophic
	38	130	208	12	20	Atrophic
	39	141	254	12	11	Atrophic
	42	146	230	19	4	Atrophic
	46	167	221	17	28	Atrophic
	47	146	197	18	6	Atrophic
	48	141	244	16	8	Atrophic
	avg.	144	236	15	9	Atrophic
Implants	0	208	2400	434	259	Normal tubules
	8	172	1550	69	22	Normal tubules
	13	137	300	9		Atrophic
	23	162	2180	338	223	Normal tubules
	25	147	179	22	13	Atrophic
	29	145	218	21	10	Atrophic
	32	140	300	20	8	Atrophic
	33	177	203	18	10	Atrophic
	35	166	259	22	9	Atrophic
	avg.	161				

In rats 0, 8 and 23 the interstitial tissue was sparse; the accessory sex organs were histologically less active than normal.

Thyroid Gland

The thyroids of the rats bearing ocular grafts were larger than those of the control hypophysectomized rats ($P < .02$) when compared on an absolute weight basis. However, on the basis of relative weight (mg./100) gm. body weight) there was only a questionably significant difference ($P < .05$). It should be noted that on this basis the hypophysectomized rat thyroids are almost twice as large as those of the intact controls (Table 1).

Microscopic examination revealed the characteristic histological picture of thyroid atrophy in all hypophysectomized rats; the presence of the implant did not significantly alter this picture.

The mean thyroid-serum iodide concentration ratio in the hypophysectomized controls was 2.3. It was significantly elevated in the rats bearing implants, though not to the concentration ratio (12.9) found in the intact animals (Table 1).

Adrenal Gland

The adrenals of the hypophysectomized rats with grafts were signifi-

cantly heavier than those of the hypophysectomized controls ($P < .01$). However, when compared on a relative weight basis (mg./100 gm. body weight), the difference was of questionable significance. ($P < .05$; Table 1).

Study of the depletion of adrenal cholesterol following stress again suggested that the implanted pituitaries were probably able to secrete ACTH. A 30% depletion of cholesterol occurred in the intact control adrenals and, as has been noted by previous investigators, a slight but significant rise in adrenal cholesterol was found in the hypophysectomized animals. The cholesterol content of the remaining adrenal in the implanted rats was either unchanged or slightly less (average of −4%). The difference between the adrenal response of the rats with implants and that of their hypophysectomized controls was statistically significant ($P < .01$).

Testes

Of the 9 graft-bearing rats that were autopsied three had testes of approximately normal weight. (Another rat which had proved fertile by

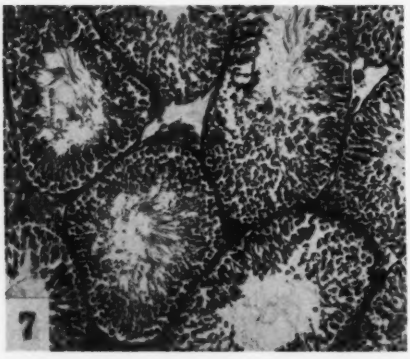

FIG. 7. Section of testis from hypophysectomized rat bearing an intraocular implant. Note the maturation of the germinal epithelium with presence of mature spermatozoa in the lumina. The interstitial tissue is much less abundant than in the testis of a normal rat. This rat was fertile. Hematoxylin and eosin; ×90.

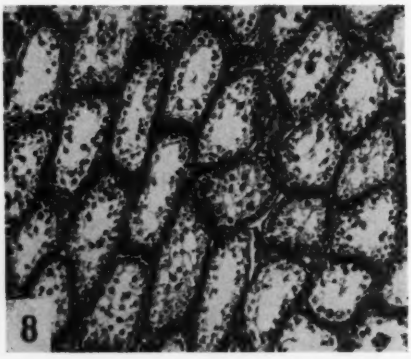

FIG. 8. Section of testis from hypophysectomized rat with intraocular implant. In contrast to the above gland (Fig. 7), this gland is indistinguishable from that of a hypophysectomized rat without an implant. Hematoxylin and eosin; ×90.

mating died prior to termination of the experiment). These gonads possessed fully developed germinal epithelium and mature spermatozoa (Fig. 7). The Leydig cells were sparse when compared to normal testes. The testes from the remaining animals were morphologically identical in all respects, irrespective of the presence of the graft, i.e., they showed atrophy of the germinal epithelium and interstitial cells (Fig. 8).

Prostate and Seminal Vesicles

The gross weights of the prostate and seminal vesicles clearly reflected the status of the testicular tissue (Table 2). Microscopically the larger glands contained epithelial tissue which was plainly stimulated, although it was not nearly so active (cell height, secretion) as normal prostatic or

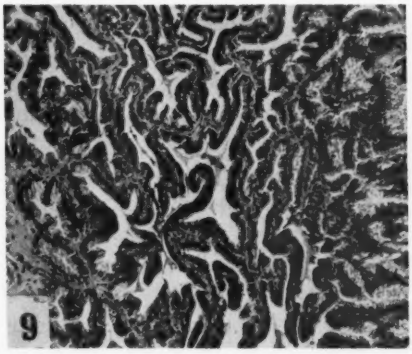

FIG. 9. Prostate from hypophysectomized rat bearing an intraocular implant and possessing mature testicular germinal epithelium. Note the relatively high degree of activity in the secretory epithelium. This tissue is less active histologically than normal prostate but remarkably different from that of the rats with inactive Leydig cells. Hematoxylin and eosin; ×90.

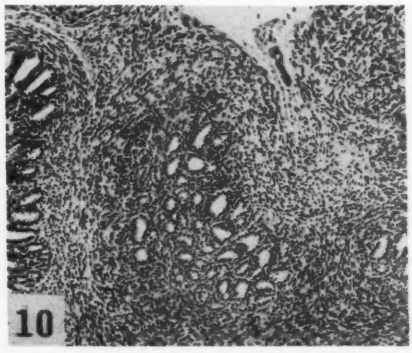

FIG. 10. Prostatic tissue from hypophysectomized rat bearing an intraocular pituitary and showing testicular atrophy. Note the glandular atrophy, which is identical with that seen in a hypophysectomized rat not bearing implants. Hematoxylin and eosin: ×100.

vesicular tissue (Figs. 9 and 10). The prostate and seminal vesicles from the implanted rats, which did not exhibit testicular response were atrophic.

DISCUSSION

It is of interest that when hypophyseal tissue from fetal rats is implanted into the eyes of hypophysectomized rats, all three major lobes "take" and actively proliferate. It was not surprising to find, in view of the dependence of the posterior lobe upon the integrity of its neural relation to the hypothalamus, that the implanted neurohypophyseal tissue was composed of numerous "glial-type" cells embedded in a fibrillar matrix, without intercellular substance positive to aldehyde-fuchsin. This situation seems analogous to the observations of Hild (9) on the structure of explants of posterior lobe tissue. It is of some import to note that the presence or amount of the posterior and intermediate lobe tissue was not related to the structure or apparent function of the anterior lobe.

Numerous previous investigators have noted the presence of but few chromophilic elements in transplanted anterior lobe tissue, as was the

case in the material studied here. It is possible that the very slight secretion of thyroid hormone by the atrophic thyroid is at least one of the limiting factors determining the granulation in the alpha cells (acidophils). In graft-bearing hypophysectomized rats given propylthiouracil the alpha cells are completely degranulated (unpublished observations). Study of implants in thyroxine treated animals would be of considerable interest in this regard. In this study it was found that thyroxine administration for a period of two months had no perceptible effect on behavior, appearance, or growth rate. It is possible that implants in a less "hypothyroid environment" might be better able to synthesize or secrete the various hormones. No rats at autopsy, however, had recently been treated with thyroxine.

The presence of the small angular basophils with a small negative image of the Golgi-apparatus and filled with granules positive to both periodic and acid-Schiff and aldehyde-fuchsin is of considerable interest and importance. These cells seem identical with the beta cells or thyrotrophs (10) of anterior lobe tissue located in the sella turcica. Available evidence strongly suggests that these cells are the site of synthesis of the thyroid-stimulating hormone (TSH) (10, 11) and that this aldehyde-fuchsin positive material is a reflection of TSH content (10, 11, 12). While cytological signs of increased stimulation were not noted in the thyroids of the implanted rats (as compared with those from the hypophysectomized controls), there was a significantly greater T/S ratio and a slight increase in the size of the thyroid in these animals. In view of this it seems quite likely that a limited secretion of TSH takes place in transplanted glands. From the above cytological and functional data it would appear then, that beta cells in the transplants are able to synthesize and store TSH but do not release amounts of it to the circulation sufficient to maintain normal thyroid activity.

Recently Scow and Greer (13) reported that hypophysectomized mice bearing pituitary transplants had a normal thyroidal uptake of I^{131}, a higher T/S ratio and a more active epithelium than control hypophysectomized mice. Propylthiouracil was found to stimulate markedly the thyroids of the implanted mice. It would appear that the pituitary gland in this species is less dependent on intact anatomical connections with the hypothalamus than that in the other species studied.

Von Euler and Holmgren (14) have recently demonstrated that the injection of relatively large amounts of thyroxine (2 μg.) directly into the anterior pituitary of the rabbit inhibited the thyroidal release of I^{131}. No effect was obtained with injection into the hypothalamus or the tuber cinereum. They also observed that ocular implants of pituitary in hypophysectomized rabbits seemed to maintain thyroidal I^{131} release at a rate similar to that of intact controls. Thyroxine administered to such implant-bearing rats inhibited release as did enucleation (at a considerable interval after I^{131} administration). Corticoid administration did but cold-

stimulation or "stress" did not inhibit release in the implant-bearing rabbits.

It would appear, therefore, from the results of all these experiments, that a simple feed-back relationship does not adequately describe the thyroid-pituitary interplay. There now can be no doubt that adenohypophyseal tissue, anatomically separated from the central nervous system, is able to synthesize and secrete thyrotropin hormone and that this activity can be checked by high levels of circulating thyroid hormone, or at least by the effects thereof. It is also clear that the thyrotrophs probably require a direct stimulus from the central nervous system in order to synthesize and release TSH much in excess of this "basal" amount.

The slightly larger adrenal glands and the difference in the response of the adrenals to acute stress, as judged by depletion of adrenal cholesterol, reflects secretion of ACTH by the transplants. These findings confirm the observations of numerous investigators, (reviewed in detail by Harris, 2) that ocular transplants of pituitary glands, while unable completely to maintain adrenal weight, are capable of secreting ACTH in response to stress.

Whether or not the slightly greater growth of the implant-bearing rats was related to secretion of growth hormone is not clear. The descent of the testes, maturation of the germinal epithelium, and fertility are as yet very difficult to assess. It has been demonstrated (15) that the ocular transplant of pituitary glands fails to support cyclic activity in the female, such animals having atrophic gonads. A number of investigators have reported testicular maturation in animals bearing transplants (16, 17 and others). It appears from the material in these studies that the secretion of FSH had been adequate for maturation of the germinal epithelium but that the secretion of LH had been deficient as judged by the appearance of the Leydig cells and the condition of the accessory sex glands. The significance of this is still obscure. There is no cytological evidence of gonadotrophic activity in the implants. The gonadotrophs certainly did not show evidence of hyperactivity in response to low steroid output by the atrophic gonads. It is, of course, quite possible that they secrete enough FSH to stimulate the testis, but that this in conjunction with difficulties in LH (and LTH) secretion is to no practical avail in the female. The presence of the residual intracranial tissue in most of the animals (composed of cells which are difficult to identify) adds an important element of uncertainty to the data presented here. Similar observations have been made by Green (18).

More adequate evaluation of these data would be aided by studies of the effects of enucleation of the implanted eye of transplanted animals with functioning testicular tissue. Similarly, the assay of pituitaries removed from the eye for thyrotrophic hormone activity will be of importance in assessing whether the observed aldehyde-fushcin positive material actually is a reflection of TSH content.

SUMMARY

Fetal pituitary glands were implanted into the anterior chamber of the eye of hypophysectomized male rats. Viable, proliferating, highly differentiated tissue representing all three pituitary lobes was observed. The anterior lobe, though composed largely of chromophobic elements, contained sparsely granulated alpha cells (acidophils), well granulated beta cells, and rare pale staining delta cells.

Growth of implant-bearing hypophysectomized rats was slightly greater than that of controls without implants.

A questionably significant increase in the size of the thyroid gland without histological activation and a marked elevation of the T/S ratio reflected slight secretion of TSH by the implants.

The presence of aldehyde-fuchsin positive granules in the implanted anterior lobe beta cells suggests that these cells may synthesize and store TSH. Release to the circulation may be defective owing to lack of the proper stimulus to the implanted beta cells.

Evidence of slight secretion of ACTH by the implants was obtained from studies of adrenal weight and depletion of adrenal cholesterol following acute stress.

Spermatogenesis (and consequent fertility) coupled with some stimulation of testicular interstitial tissue and accessory sex glands indicated that some implants were secreting FSH and LH.

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TESTOSTERONE-INDUCED N-RETENTION IN CASTRATED RATS ON DIETS CONTAINING INCOMPLETE PROTEINS[1]

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SEVERAL important investigations have been published recently on the mechanism of the biological synthesis of proteins, but very little has been contributed to our knowledge of the intrinsic factors which initiate and determine the extent of tissue protein synthesis. It is usually assumed that both nervous and hormonal factors govern protein anabolism i.e. the protein synthesis for growth and repair. Based on the fundamental discovery by Kochakian and Murlin (1) that testosterone increases nitrogen retention, androgens are presently regarded by many authors as particularly potent anabolic agents. In connection with our investigations on protein anabolism, we studied, therefore, the mechanism of androgen-induced nitrogen retention.

Earlier we found that the speed and degree of protein depletion and of subsequent repletion in *normal* rats is not influenced by testosterone treatment (2).

In further investigations (3) on normal young men on restricted sodium intake, it was found that the net weight gain and water retention during the first few days following hormone administration were out of proportion to the relatively small amount of nitrogen retention. The ratio of potassium to nitrogen retained was also considerably higher than that found in normal adult tissue. After testosterone was withdrawn, all subjects lost weight and water, despite the fact that the net nitrogen balance remained positive for a few days.

These observations and similar published findings (4) raised the possibility that the nitrogen retention observed after androgen treatment in normal men and animals may not represent real tissue protein synthesis.

Therefore, in the following experiments we investigated: 1) The effect of testosterone on the nitrogen balance of rats kept on zein and on gelatin diets which, lacking one or more amino acids, do not support protein synthesis; and 2) The effect of testosterone on rats receiving in their diet a mixture of three non-essential amino acids as a sole nitrogen source. This

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was to test the possibility raised by some authors that the testosterone effect may be due to increased tubular reabsorption of nitrogen in the kidney (5).

METHODS

Adult male rats of the Wistar strain were used. The animals, with the exception of those in Experiment 3, were castrated several weeks before the experiment. The rats received Rockland Rat Diet before and during the intervals between experiments.

Experimental Diets: 1. Basal Diet 85 gm., plus 15 gm. zein; 2. Basal Diet 85 gm., plus gelatin 15 gm; 3. Basal Diet 85 gm., L-Glycine 3.4 gm., L-Alanine 4 gm., and L-Sodium Glutamate 7.6 gm. The composition of the experimental basal diet was as follows: Corn Starch 60 gm., Cane Sugar 8 gm., Butter Fat 10 gm., Salt Mixture (U.S.P. #2) 5 gm., Corn Oil 2 gm., Choline HCl 0.2 gm. All the necessary vitamins were added to the same extent as used in earlier experiments (2).

Two days before the start of the nitrogen balance experiments the rats were put in individual metabolism cages. Urine and feces were collected daily and the nitrogen content was determined according to the Folin-Nessler method (6). The testosterone propionate (TP) (Lilly) was injected subcutaneously. Control animals received 0.1 ml. sesame oil subcutaneously.

RESULTS

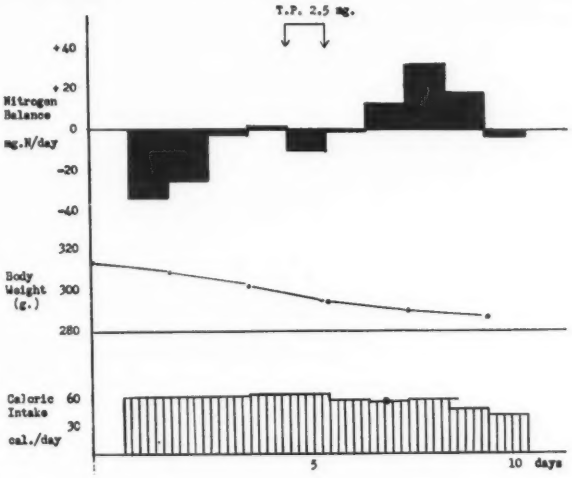
Figure 1 shows the effect of the TP injection on the nitrogen balance of 11 *castrated* male rats which received a diet containing zein as the sole nitrogen source, and were, therefore, in negative nitrogen balance. On the fourth and fifth days after the start of the experiments, each rat received subcutaneously 0.1 ml. of a 25 mg./ml. solution of TP in oil, thus making the total dose 5 mg. of TP. Following the last injection the urinary nitrogen excretion decreased and the nitrogen balance became positive for three days. On the last day of the experiment the nitrogen balance was again negative.

The lower column in Figure 1 indicates the caloric intake of the animals. The curve of the body-weight shows that in spite of the positive nitrogen balance the animals continued to lose weight after TP injection.

In a *control* experiment 9 *castrated* male rats fed the zein diet were injected with 0.1 ml. of sesame oil on the fourth and fifth days of the experiment. The nitrogen balance was not affected by the oil injections.

In further experiments the effect of TP (total dose 5 mg. per rat) on the nitrogen balance of 4 *normal* rats on the zein diet was investigated. In this experiment the androgen did not affect the balance. This result is in agreement with that published by Kochakian, Moe, and Dolphin (7), who found testosterone less effective on the nitrogen balance of normal male rats than on that of castrated animals.

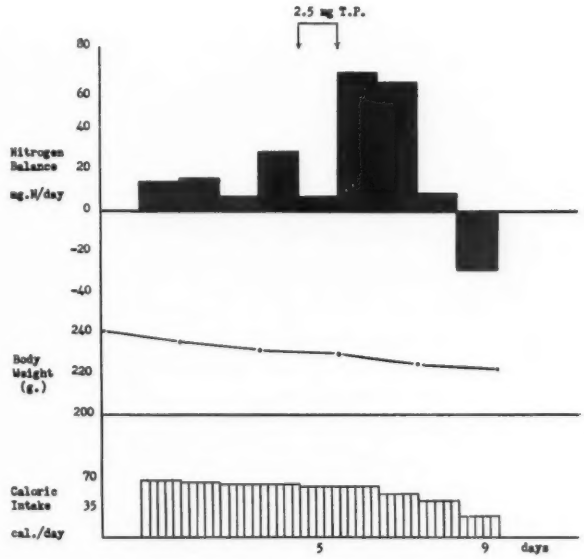
In the second group of experiments, *castrated* animals received a diet containing *gelatin* as the sole nitrogen source. Data of the experiments with 4 animals are tabulated in Figure 2. In the initial control period the nitrogen balance of rats on the gelatin diet was slightly positive. After injection of hormone the nitrogen retention increased considerably.



Effects of subcutaneous administration of Testosterone Propionate on Nitrogen balance of castrated male rats fed zein diet.

Total number of animals: 11

FIG. 1



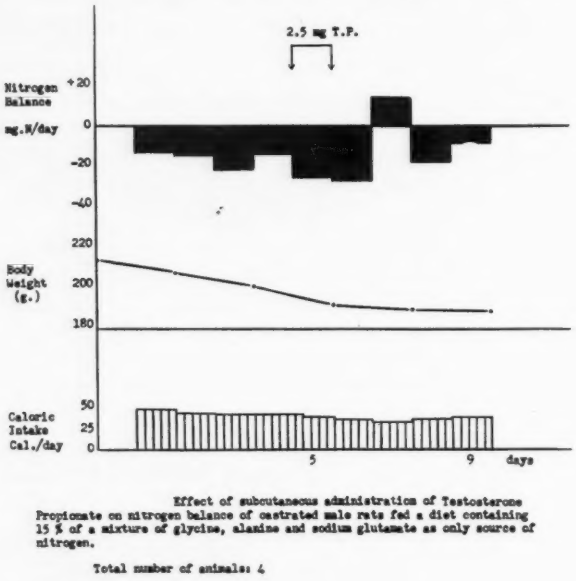
Effect of subcutaneous administration of Testosterone Propionate on nitrogen balance of castrated male rats fed gelatine diet.

Total number of animals: 4

FIG. 2

In another experiment the animals received TP injections for seven consecutive days. Prior to receiving the injections, the castrated rats retained some nitrogen on a gelatin diet. The retention was considerably increased during the seven days of hormone treatment.

In the final group of experiments a mixture of *3 non-essential amino acids*



Effect of subcutaneous administration of Testosterone Propionate on nitrogen balance of castrated male rats fed a diet containing 15 % of a mixture of glycine, alanine and sodium glutamate as only source of nitrogen.

Total number of animals: 4

FIG. 3

was fed as the sole source of nitrogen. Figure 3 shows a slight but definite nitrogen retention on the day following the last hormone injection.

DISCUSSION

Our experiments show that TP treatment increases the nitrogen retention even in cases where zein or gelatin are the only sources of dietary nitrogen. These proteins lack one or two essential amino acids and, therefore, normally do not support protein synthesis. Munro and Chalmers (8) also fed zein in their investigations of the mechanism of nitrogen retention during growth hormone treatment of rats. The authors found that injection of growth hormone decreased nitrogen excretion, and that nitrogen retention was the same whether or not zein diet was supplemented with the missing essential amino acids. Their conclusion in regard to the growth hormone was that "the mechanism causing nitrogen retention is not necessarily associated with the process of growth."

Our results with feeding proteins deficient in some amino acids seem to suggest that nitrogen retention due to TP does not necessarily indicate

tissue protein synthesis. It is conceivable that in the experiments in which tryptophan-free gelatin was fed, TP promoted the deposition of tryptopan-free collagen in the bones. The zein diet is, however, deficient in tryptophan, lysine, and other essential amino acids (9), and thus could not supply the "building stones" for collagen synthesis. We have to consider, however, the possibility that because of the anabolic effect of TP, some of the essential amino acids which are normally catabolized as a result of "wear and tear" of body proteins are re-utilized in order to supplement the incomplete supply of dietary amino acids. We are presently investigating the question of how and where the nitrogen is retained.

When rats were fed a diet containing a mixture of 3 non-essential amino acids in an otherwise protein free diet, there was a slight increase of nitrogen retention which was, however, much smaller than that observed in those fed zein or gelatin. The results of these last experiments may indicate that at least one part of the TP induced nitrogen retention is independent of protein synthesis, and is the result of decreased renal excretion of nitrogen-containing metabolites.

SUMMARY

Testosterone propionate treatment increased the nitrogen retention of castrated adult male rats even when the diet contained zein or gelatin as the only nitrogen sources, compounds which do not support protein synthesis because of their lack of some essential amino acids.

A slight shift to positive nitrogen balance has been observed in TP-treated rats which received, as their only nitrogen source, a mixture of 3 non-essential amino acids. The results, therefore, seem to suggest that TP-induced nitrogen retention does not necessarily indicate an increase of tissue protein synthesis.

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HISTOPHYSIOLOGY OF THE ADRENAL CORTEX IN DOGS WITH MILD AND SEVERE CARDIAC DAMAGE[1,2]

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PATIENTS with uncompensated congestive heart failure exhibit conspicuous salt and water retention with ascites and edema. It is possible to reproduce typical right-sided cardiac failure in dogs by combining damage to the tricuspid valve with stenosis of the pulmonary artery (1, 2). Damage to the tricuspid valve alone, while causing no overt salt and water retention, does increase venous pressure and total exchangeable sodium and decrease the ability to excrete a salt load (3, 4). Because of this disturbed control of salt and water excretion, we have compared histologically the adrenal glands from dogs with mild valvular damage and from dogs with frank congestive heart failure with those from normal dogs to determine whether or not the adrenal cortex undergoes any histological alterations in association with the production of cardiac insufficiency.

METHODS

Ten experimental dogs, used over periods of months to study other parameters of cardiac insufficiency, were killed and their adrenal glands were fixed for study. Five animals had mild tricuspid insufficiency of 3–12 mo. duration. Five had tricuspid insufficiency (1–7 mo.), followed by pulmonary artery stenosis (1–6 mo.): two were in frank failure but not moribund when killed, two were in the terminal stages of failure, and one had begun a spontaneous diuresis 21 days previously (see Table 1). The adrenals from five normal dogs kept under the same conditions were taken for comparison.

All the dogs were adults weighing approximately 20 kg. They were fed a standard diet that was adequate to maintain constant weight in the normal state. The sodium intake per day was about 57 m. eq. and the potassium intake about 45 m. eq.

Tricuspid insufficiency was produced by inserting a valvulotome through the right auricular appendage and cutting the chordae tendineae (1). In animals with this lesion, the resting right atrial pressure rises from about zero to 75–125 mm. H_2O (2), and resting cardiac output is in the lower range of normal (100–150 ml./kg./min.). Such dogs can exercise on a treadmill quite normally and fail to develop frank failure even after months of exhausting daily exercise (2). Plasma volume is increased 10–20% (23). Total body

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sodium and chloride rise to levels somewhat above normal, as indicated by determinations of exchangeable sodium and of cumulative sodium and chloride balances (4). Excretion of sodium and water following an intravenous injection of physiological saline is moderately delayed (3).

At least one month after the tricuspid damage, five of the dogs were subjected to a second operation in which the diameter of the pulmonary artery was reduced to about one-half of normal (1). Such dogs promptly develop typical right-sided congestive heart failure, with cardiac enlargement, hepatomegaly, an increase in right atrial pressure to 250–300 mm. H_2O (2), and a decline in cardiac output to 75–100 ml./kg./min.—terminally it falls even lower. There is obvious salt and water retention, resulting in ascites and, later, edema. Exchangeable sodium and the cumulative sodium chloride balance rise steadily (4). Such dogs are unable to excrete an intravenous salt load, although the basal glomerular filtration rate may be normal (3). Serum sodium and potassium levels are normal (Na, 145 m. eq./L.; K, 4.5 m. eq./L) until the terminal stages, when the ratio drops. At this terminal stage, marked renal vasoconstruction develops, the glomerular filtration rate falls and dyspnoea occurs.

A median segment of one of the adrenal glands from each dog was fixed for at least a week in 10% neutral buffered formalin, washed overnight and sectioned at 15 μ on a freezing microtome. Sections were stained for lipides in a saturated solution of Sudan black B in 70% ethanol, 1 and 7 minutes, and then mounted in glycerol-gelatin. Other sections were stained for pseudoplasmals in the Schiff reagent, 30 minutes (15), and for cholesterol by the Schultz reaction.

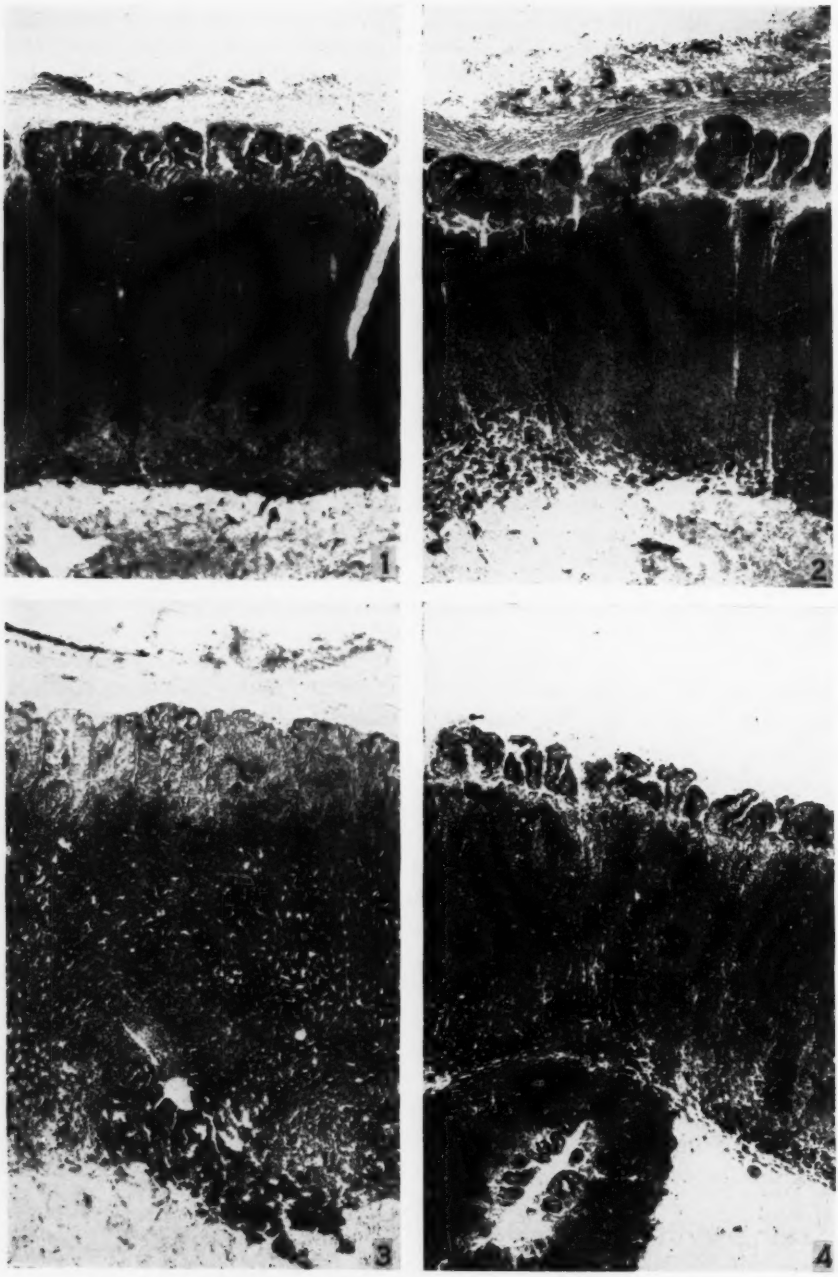
The average thicknesses of the cortical zones were determined by projecting midsagittal sections at 34× and measuring them at 10 different radii.

OBSERVATIONS

The average thickness of the zona glomerulosa and of the inner cortical zones (fasciculata and reticularis) for each of the dogs is presented in Table 1.

The adrenal cortex in the five control dogs possessed a zona glomerulosa composed of narrow arcades; in some areas the arcades tended to lie flat, in others they stood upright (Fig. 1). The remaining cortex consisted of a broad zona fasciculata and a narrow zona reticularis. The cells in all of the zones contained abundant lipide droplets. The droplets were generally small except in the reticularis, where they were large and sometimes consisted of lipide pigments. The droplets in the glomerulosa reacted only moderately with the Schiff carbonyl reagent, those of the outer fasciculata reacted strongly, while those of the inner fasciculata and reticularis were virtually unstained. This test provides a rough gauge of the concentration of unsaturated lipides in the droplets; in the rat, at least, this concentration is positively correlated with secretory rate (15). The droplets failed to give a positive Schultz cholesterol test in the specimens from the normal animals (or from the experimental animals), indicating a relatively low concentration.

The glands from all five dogs with tricuspid insufficiency showed enlargement of the glomerulosal arcades; the cells appeared hypertrophied so that the arches were both fuller and taller (Fig. 2). The inner zones appeared unaffected. The cells in the glomerulosa and outer fasciculata con-



FIGS. 1–4. Frozen sections of dog adrenals stained with Sudan black B for 7 minutes; all ×45.

TABLE 1. AVERAGE THICKNESS OF ADRENOCORTICAL ZONES IN CONTROL AND EXPERIMENTAL DOGS

Dog	Thickness, mm.	
	Glomerulosa	Inner zones
*C1, ♂	0.12	1.15
C2, ♀	0.13	1.15
C3, ♀	0.14	1.20
C4, ♂	0.16	1.01
C5, ♀	0.16	1.11
	av. 0.14	av. 1.12
TI1, ♀	0.22	1.07
TI2, ♀	0.23	1.34
TI3, ♂	0.24	1.18
TI4, ♀	0.24	1.04
TI5, ♀	0.29	0.84
	av. 0.24	av. 1.09
TI&PS1, ♀ FF	0.28	1.59
TI&PS2, ♀ FF	0.36	1.11
TI&PS3, ♀ T	0.23	1.26
TI&PS4, ♂ T	0.33	1.88
	av. 0.30	av. 1.46
TI&PS5, ♂ SD	0.16	1.11

* Abbreviations: C, control; TI, tricuspid insufficiency; PS pulmonary artery stenosis; FF, frank failure; T, terminal; SD, spontaneous diuresis.

tained abundant small lipide droplets that gave the Schiff reaction to the same degree as in the control glands.

The adrenals from the four dogs in frank failure all showed similar or more marked hypertrophy of the zona glomerulosa. Three (excepting TI & PS 3) also showed depletion of lipides from this zone (Fig. 3). Two of the animals exhibited considerable enlargement of the inner zones of the cortex, and one dog killed when moribund (TI & PS 4) had depletion of lipides from the inner zones as well.

One dog in failure developed a spontaneous diuresis (TI & PS 5). Over a period of about three weeks he lost 10 kg. and was then killed. The cortex of this animal appeared indistinguishable from that of a normal dog (Fig. 4).

FIG. 1. Control dog, C3.

FIG. 2. Dog with damage to the tricuspid valve for over a year, TI1. The glomerulosal arcades are hypertrophied.

FIG. 3. Dog with tricuspid damage for a total of 6 months and superimposed pulmonary artery stenosis for 3 months, TI&PS2. The glomerulosal arcades exhibit further hypertrophy as well as a lipide depletion.

FIG. 4. Dog with congestive heart failure of a year's duration that developed a spontaneous diuresis 3 weeks before being killed, TI&PS5. The adrenal cortex appears normal.

DISCUSSION

The anatomical and physiological dichotomy of the two major zones of the dog adrenal cortex has been recognized for about a quarter of a century. In 1933, Houssay and Sammartino (14) reported that while hypophysectomy causes severe atrophy of the inner cortex (fasciculata and reticularis), it produces no conspicuous alteration of the glomerulosa. This observation, coupled with the fact that hypophysectomized animals do not suffer from the electrolyte disturbances characterizing adrenalectomized animals, provided Swann (25) with the foundation of the hypothesis that the zona glomerulosa is relatively autonomous of the anterior pituitary gland and produces the hormone(s) affecting electrolyte balance, whereas the inner cortex is under the control of pituitary corticotrophin and produces the hormone(s) influencing protein and carbohydrate metabolism.

In the last few years, since the identification of aldosterone, more direct evidence for this dichotomy has been forthcoming. Notably, Farrell, Rauschkolb and coworkers (10, 20), by measuring hormone output into the adrenal vein, found that hypophysectomy of dogs depressed the secretion of 11-oxygenated corticoids about 90% but that of aldosterone only about 33%. In such dogs, the administration of corticotrophin augmented the secretion of the 11-oxygenated hormones but not that of aldosterone. In a variety of species, lowering the ratio of administered sodium/potassium appears to enhance aldosterone output, whereas raising the ratio depresses it (dog: 16, 22; rat: 9, 13, 24; human being: 17, 18; isolated bovine gland: 21). These effects are observed even in the absence of corticotrophin (18, 21).

According to the observations reported here, the only consistent histological alteration in the adrenal cortex in animals with mild cardiac insufficiency is enlargement of the glomerulosal cells. This constitutes a sign of moderate hyperactivity (7). Usually, frank failure induces further enlargement of the cells, coupled, in some instances, with a loss of free lipides. Such depletion of enlarged cells is a sign of marked hyperactivity. Only irregularly is there any conspicuous enlargment and depletion of the fasciculata. Hamilton *et al.* (12) have recently reported similar results.

Although our histophysiological evidence thus suggests that there is increased aldosterone output in chronic cardiac insufficiency, there is still conflicting physiological evidence on this question. Thus Luetscher and Johnson (17) found that urinary aldosterone levels were generally above normal in cardiac patients having ascites and edema, and Gordon (11) reported that the level remained supranormal even after compensation. Muller *et al.* (19), on the other hand, have pointed out that not all patients with uncompensated heart failure have hyperaldosteronuria—especially if they have not been treated by salt restriction. Liddle *et al.* (16a) found the level always supranormal in patients with congestive heart failure given a diet containing ample salt. For dogs, Davis and coworkers have

provided evidence that cardiac failure results in an increased aldosterone output, as measured either in the urine (5) or adrenal venous blood (6). The output appeared to be only slightly enhanced in the first 10 days after operation, in which period ascites was developing. A study in Farrell and Berne's laboratory (8) has not confirmed these findings in dogs in which failure was produced in the same way; these animals, however, were studied within the first two weeks after operation.

Should hyperaldosteronism prove to be a usual concomitant of chronic congestive heart failure, the available evidence still leaves unanswered the following questions: i, whether the putative hyperaldosteronism precedes and causes the salt retention, or develops secondarily and is permissive in character; ii, what stimulates the zona glomerulosa in the absence of detectable alteration in serum electrolyte concentrations and, in experimental animals at least, in the presence of normal intake of sodium and potassium.

SUMMARY

Dogs with mild valvular damage of the right heart, characterized by slight increase in total exchangeable sodium and a reduced ability to excrete sodium chloride, showed moderate enlargement of the adrenal zona glomerulosa. Dogs with superimposed stenosis of the pulmonary artery, which exhibit typical cardiac failure with marked salt and water retention, sometimes showed further enlargement of this zone and a loss of its lipide droplets. One such dog that developed a spontaneous diuresis three weeks previously had an entirely normal adrenal cortex.

The enlargment of the glomerulosa presumably signifies enhanced production of aldosterone in chronic cardiac insufficiency. The results do not help to resolve the questions of whether increased levels of aldosterone are primarily responsibile for the sodium retention and what stimulates the zona glomerulosa.

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INCREASED GROWTH PROMOTING SUBSTANCE IN THE PLASMA OF PREGNANT RATS[1]

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THE presence of growth promoting substances in blood has been demonstrated by Westman and Jacobsohn (1) and Van Dyke *et al.* (2) by cross transfusion between parabiotically united normal and hypophysectomized rats. The hormone or hormones carried in the cross circulation from the intact rat resulted in growth of the hypophysectomized partner. Since that time attempts were made to demonstrate growth hormone-like activity in the human plasma by Kinsell *et al.* (3), Segaloff *et al.* (4), Gemzell *et al.* (5), and Greenspan (6), also in pregnant goat plasma by Cotes and Young (7), and in young pig and calf plasma by Gemzell *et al.* (5). It is the purpose of this paper to extend the observations already reported (8) concerning the presence of growth promoting substance(s) in the plasma of normal rats and the increase during pregnancy.

MATERIAL AND METHODS

Blood was collected from normal female rats of the Long-Evans strain 90–120 days of age and from pregnant rats of the same age.[2] The pregnant rats were bled at different periods between days 5 and 21 of pregnancy.[3] The blood was drawn from the aorta and centrifuged immediately under cover of mineral oil for 12 minutes at 3000 rpm. The plasma was then aspirated into a syringe and injected into recipient hypophysectomized rats,[4] the time consumed being less than 30 minutes after the time of sacrifice of the donor rats. Graded doses of plasma, 1–6 cc., were injected into the test animals. Each experiment was repeated at least twice.

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[1] Aided in part by USPH Grants A-661(C) and RG-4965.

[2] All normal and pregnant rats were maintained on a modified McCollum Diet I, the diet used in the breeding colony, consisting of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride iodized 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (vit. A and D concentrate) 1%, supplemented by lettuce.

[3] Female rats at the proestrous stage of their estrous cycle are individually caged overnight with male rats. The next day they are checked for sperm in the vaginal smear and if positive this day is considered day 0 of pregnancy.

[4] All hypophysectomized rats were maintained on the following diet: ground whole wheat 68.5%, casein 5.0%, alfalfa leaf meal 10.0%, fish oil 5%, sodium chloride 1.5%, KI solution added (450 mg. KI per liter, 300 cc. per 270.5 lbs. of diet). In addition a wet mash of the previously described McCollum Diet was offered every afternoon. All rats received a supplement of lettuce 3 times weekly. They were maintained at 74° ±1° F° temperature.

Female rats of the Long-Evans strain were hypophysectomized at 26–28 days of age and were injected with the normal or pregnant rat plasma after a post-operative period of 12–14 days. Half of the control hypophysectomized rats were injected with saline and half were left uninjected. Since no differences were observed in their responses all values are combined as control values. The injections were given intraperitoneally and were continued for periods of either four or twelve days. At the end of the experimental periods the animals were sacrificed and the tibia, thyroid, and thymus were dissected for examination. The completeness of hypophysectomy of the recipients was judged by the examination of sella turcica and by the weight and histological appearance of the pituitary dependent organs. No animals found to be incompletely hypophysectomized are included in the results listed. The rats injected for twelve days and their corresponding controls were weighed every 4 days and the tail length was measured at the beginning and the end of the experimental period. A minimum of 4 rats was used for each assay level.

The tibias were stained according to the method of Evans *et al.* (9) and Greenspan *et al.* (10). The width of the uncalcified portion of the proximal epiphyseal cartilage of the tibia was measured under the low power of the microscope using a micrometer eyepiece.

In order to obtain further confirmation of the growth promoting potency of plasma the body weight and tail length increases were measured as well as the increase in width of the tibial epiphyseal disc, in the rats which had received injections of plasma for 12 days.

To assist in interpretation of the growth promoting potency of the plasma a standard dose-response curve was made using a purified pituitary bovine growth hormone preparation (11). The average response of groups of 5 to 8 rats after receiving growth hormone in total doses varying from 4 to 140 μg. over a 4-day period showed a straight line relationship between the logarithm of the dose and cartilage width when plotted on semi-log scale (Fig. 1). That slope of the curve ($Y = 180.04 + 78.9 \log X$) is not different from that described by Greenspan *et al.* (10).

Sixteen hours before the sacrifice, the recipient hypophysectomized rats were injected with 0.5 μc. of I^{131} and at autopsy the thyroids were fixed in Bouin's fluid and counted in a scintillation counter (12). Afterwards they were sectioned and stained with hematoxylin-eosin.

RESULTS

Table 1 presents the data obtained after the administration of normal female rat plasma for four days into hypophysectomized rats. Injection of one cc. of plasma daily for four days did not result in a significant increase in the width of the proximal epiphyseal cartilage of the tibia, however, doses of 2 cc. or more increased tibial width to 200 micra or more, values considered significant in tests for the presence of pituitary growth hormone. The highest value, 224 micra, was obtained at a dose of 6 cc. Figure 2 shows that the mean response from each dose level plotted on a semilogarithmic scale resulted in a straight line where $Y = 163.2 + 80.1 \log X$. Table 2 shows the evidence obtained after injection for 12 days for growth promoting substances in normal plasma. A daily dose of 3 cc. resulted in an increase in the body weight of 5 gm. and a tail length increase of 3 mm. At higher levels of plasma a weight increase of 10 gm. and a tail increase of 4 mm. resulted. The thymus weight increased as it does from growth hormone

TABLE 1. EVIDENCE FOR GROWTH PROMOTING FACTOR IN NORMAL FEMALE RAT PLASMA ASSAYED IN HYPOPHYSECTOMIZED RATS, 4-DAY TEST

Daily dose	No. of rats	Tibial cartilage width	Body weight increase	Thymus weight	Thyroid	
					I^{131} uptake	weight†
cc./da./4-da.		micra	gm.	mg.	%	mg.
1.0	4	157±3*	0.5	145± 9	—	—
1.0	6	167±4	0.7	—	.47± .09	7.5
2.0	5	190±3	1.0	155±10	.42± .05	—
2.4	6	196±5	0.7	—	.54± .10	—
2.9	6	204±5	0.0	—	.41± .09	—
3.0	6	194±6	3.3	—	.47± .08	7.1
3.0	4	203±5	2.2	—	.44± .05	—
3.5	4	205±4	1.7	—	.60± .10	7.0
3.5	5	210±7	3.0	167± 8	.23± .03	—
4.0	4	213±4	3.0	165± 4	—	—
4.1	4	210±4	5.5	175± 9	.30± .04	—
4.8	4	218±3	2.7	—	.48± .05	7.1
6.0	4	224±2	4.0	180 14	.50± .07	—
Controls	60	151±2‡	1.5	110± 8	.45± .03§	7.1

* $SE = \frac{d^2}{n(n-1)}$

† Histologically negative.

‡ The control values have outside limits between 137 and 170 micra.

§ The control values have outside limits between .25 and .62% I^{131} uptake of the injected dose.

both after 4 and 12 daily injections (Tables 1 and 2). At the doses injected there was no effect on the I^{131} uptake, weight, or histology of the thyroids of the recipient hypophysectomized rats (Tables 1 and 2).

Table 3 shows the evidence for greater growth promoting activity of pregnant rat plasma. Daily doses of 1 cc. plasma taken from pregnant rats between day 5 and 21 were sufficient to increase the tibial cartilage width

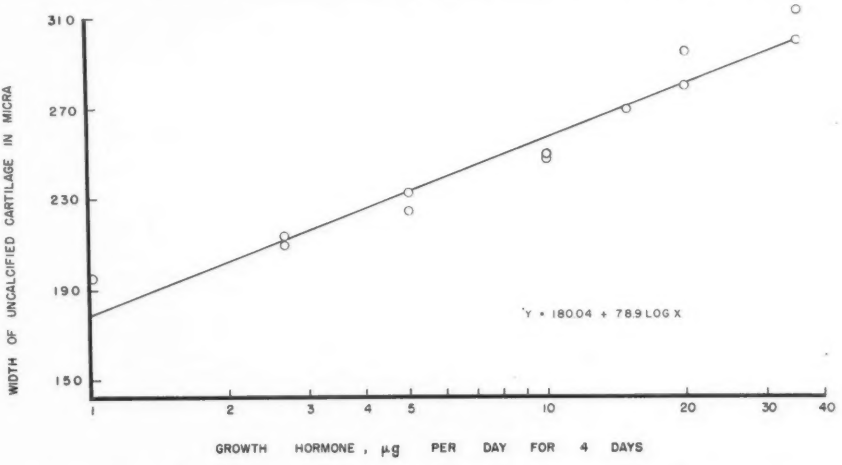

FIG. 1. Effect of purified bovine growth hormone on proximal epiphyseal cartilage of tibia of hypophysectomized rat.

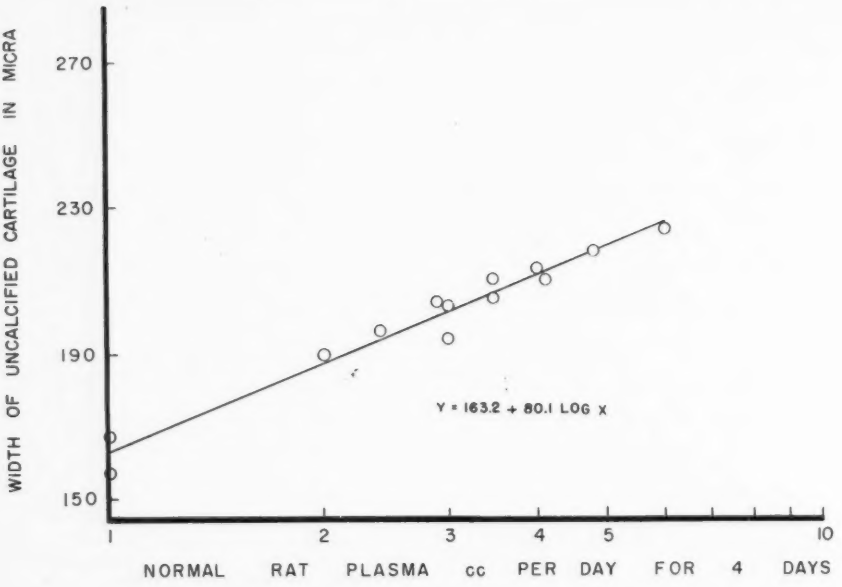

FIG. 2. Effect of normal female rat plasma on proximal epiphyseal cartilage of tibia of hypophysectomized rat.

to 185–206 micra in daily doses of 1 cc. Doses of 2 to 3 cc. increased the width to 220 micra and 5 cc. resulted in widths as high as 270 micra. The slope of the line constructed from these values ($Y = 193.8 + 82.4 \log X$) does not differ from that for normal plasma (Fig. 3).

Table 4 presents the evidence from the 12 day test for increase in growth promoting power of pregnancy plasma. A daily dose of 3 cc. of plasma from either day 17 or 21 of pregnancy resulted in an increase in body weight of hypophysectomized recipients of 10 to 13 gm., and an increase in the tail

TABLE 2. EVIDENCE FOR GROWTH PROMOTING FACTOR IN NORMAL FEMALE RAT PLASMA ASSAYED IN HYPOPHYSECTOMIZED RATS, 12-DAY TEST

Daily Dose	No. of rats	Tibial cartilage width	Body weight increase	Tail length increase	Thymus weight	Thyroid	
						I^{131} uptake	Weight*
cc./da./12-da			gm.	mm.	mg.	%	mg.
1.0	5	145±3	1.5±.7	1±.02	145±4	.41±.05	7.0
2.6	6	190±2	5.0±1	—	147±4	—	7.0
3.0	6	191±3	5.0±1	3±.01	142±3	.28±.03	—
3.0	5	197±1	7.0±1	3±.02	141±4	—	7.4
4.8	6	215±2	10.0±2	4±.01	165±5	—	8.1
5.0	6	210±3	8.0±1	3±.02	156±6	.38±.04	—
Controls	60	151±2†	2.0±.1	0.5±.02	110±8	.45±.03‡	7.1

* Histologically negative.
† The control values have outside limits between 137 and 170 micra.
‡ The control values have outside limits between .25 and .62% I^{131} uptake of injected dose.

TABLE 3. EVIDENCE FOR GROWTH PROMOTING FACTOR IN PREGNANT RAT PLASMA ASSAYED IN HYPOPHYSECTOMIZED RATS, 4-DAY TEST

Day of Pregnancy	Daily dose	No. of rats	Tibial cartilage width	Body weight increase	Thymus weight	Thyroid	
						I^{131} uptake	Weight*
	cc./da./4-da.		micra	gm.	mg.	—	mg.
	1.0	5	187 ± 5	0	—	.45 ± .03	—
5	2.0	5	203 ± 1	2	—	.37 ± .02	7.1
	4.3	5	230 ± 5	4	—	.60 ± .05	7.7
9	1.0	6	203 ± 3	0	188 ± 7	—	—
	4.8	5	267 ± 7	5	210 ± 10	—	—
	1.0	6	185 ± 3	2	—	—	—
	1.0	4	206 ± 3	3	—	.42 ± .07	7.2
	3.0	4	226 ± 3	4	—	.62 ± .10	7.1
13	3.3	5	236 ± 3	3	165 ± 5	.50 ± .10	7.1
	3.8	4	244 ± 2	3	—	.55 ± .10	—
	4.75	6	243 ± 3	4	—	.47 ± .08	—
	5.0	6	272 ± 8	4	—	.39 ± .04	—
16	1.5	5	209 ± 3	3	—	.35 ± .02	—
	2.8	6	217 ± 4	3	176 ± 7	.21 ± .05	—
18	2.0	5	219 ± 3	2	—	—	—
	3.6	6	235 ± 6	2	169 ± 4	—	7.0
	1.0	8	195 ± 4	1	—	—	—
	2.8	8	220 ± 3	3	—	.77 ± .18	—
20	3.0	8	228 ± 1	5	160 ± 5	—	7.0
	3.6	8	239 ± 5	3	—	.34 ± .03	—
	3.7	8	237 ± 2	2	—	.72 ± .20	—
	5.0	8	255 ± 3	4	—	.50 ± .08	—
	2.5	5	228 ± 3	5	—	—	—
21	3.4	6	230 ± 7	8	156 ± 3	.32 ± .05	7.4
	5.0	5	249 ± 2	3	165 ± 2	.50 ± .03	7.1
Controls	—	60	151 ± 2†	1.5	110 ± 3	.45 ± .03‡	7.1

* Histologically negative.
† The control values have outside limits between 137 and 170 micra.
‡ The control values have outside limits between .25 and .62% I^{131} uptake of the injected dose.

length of 4 to 5 mm.; a dose of 5 cc. per day increased the body weight 13 to 17 gm. and the tail length 8 mm.

The thymus weight was increased by pregnancy plasma after both a 4 and 12 day injection period sometimes as much as 100%. No evidence was found for thyroid stimulation as judged by I^{131} uptake, morphological change or weight increase (Tables 3 and 4).

DISCUSSION

The data show that growth promoting substance(s) are present in the normal rat plasma. The increase in epiphyseal cartilage width equalled

TABLE 4. EVIDENCE FOR GROWTH PROMOTING FACTOR IN PREGNANT RAT PLASMA ASSAYED IN HYPOPHYSECTOMIZED RATS, 12-DAY TEST

Day of pregnancy	Daily dose	No. of rats	Tibial cartilage width	Body weight increase	Tail length increase	Thymus weight	Thyroid	
							I^{131} uptake	Weight*
	cc./da./12-da.		micra	gm.	mm.	mg.	%	mg.
	2.0	6	219 ± 6	10 ± 1	—	154 ± 6	—	7.0
17	3.0	5	232 ± 5	15 ± 1	5 ± .2	183 ± 7	—	7.6
	5.0	6	270 ± 7	17 ± 1	8 ± .3	196 ± 4	.45 ± .08	8.0
	1.0	6	185 ± 5	4 ± .5	3 ± .1	144 ± 3	.56 ± .07	—
21	3.0	6	230 ± 7	10 ± 1	4 ± .2	156 ± 5	.32 ± .08	—
	5.0	6	260 ± 6	13 ± 1	8 ± .4	142 ± 4	.39 ± .07	—
Controls	—	60	151 ± 2†	2 ± .1	0.5 ± .02	110 ± 8	.45 ± .03‡	7.1

* Histologically negative.
† The control values have outside limits between 137 and 165 micra.
‡ The control values have outside limits between .25 and .62% I^{131} uptake of injected dose

and exceeded that considered significant in evaluating pituitary growth hormone by the 4-day tibia test. When the plasma was given over a longer period of time (12 days) it resulted in significant increases in body weights and tail lengths. The ability of the plasma to enhance growth was greater during pregnancy. A straight line relationship could be demonstrated for growth promoting potency for both normal and pregnancy plasma between the response and the logarithm of the dose, the slope of the curve being approximately the same as obtained from pituitary growth hormone (Fig. 1–3).

When the extent of the growth resulting from injection of normal or pregnant rat plasma is compared with that obtained from the administra-

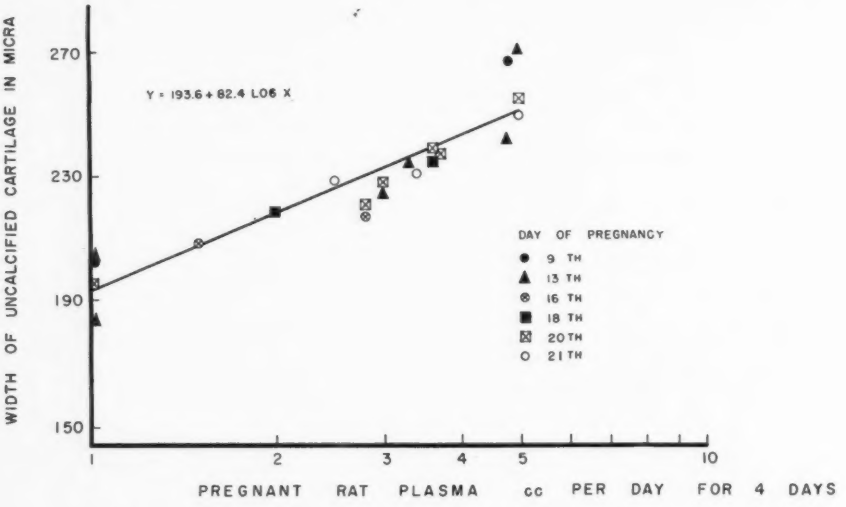

FIG. 3. Effect of pregnant rat plasma on proximal epiphyseal cartilage of tibia of hypophysectomized rat.

tion of purified pituitary bovine growth hormone it is found that a total dose of 8 cc. of normal rat plasma gave the same tibial response as a dose of 8 μg. of purified growth hormone, or that an equivalent 1.0 μg. of growth hormone was present per cc. of normal plasma. Judged by the criterion of increase in tail length (in a 12-day test period, Fig. 4 and Table 2) a daily dose of 3 to 5 cc. of normal rat plasma produced approximately the same response as 7.5 μg. of the pituitary growth hormone or an equivalent of 1.6 to 2.5 μg. of growth hormone per cc. of normal plasma.

The pregnant rat plasma judged on the basis of the 4-day tibia test contained between day 9 and 21 the equivalent of 2.5 to 3.5 μg. per cc. of purified growth hormone. On day 5 only 1 μg. to 1.3 μg. per cc. were found to be present, the value was therefore similar to that of the nonpregnant

female rat. Calculations based on tail length increase over 12 days, similarly indicated a 3-fold increase in growth promoting potency of plasma during pregnancy (Fig. 4, Table 4).

The values for growth promoting substances in normal plasma given here agree favorably with those indicated by Van Dyke *et al.* (2). In the data presented in that paper, blood from normal rats was allowed to pass into hypophysectomized rats for 16 days at 33 days of age. The growth of the normal young rats, from 33 to 49 days of age corresponded on the standard dose-response curve to the growth which can be induced in hypophysectomized assay animals by injection of 140 μg. per day of

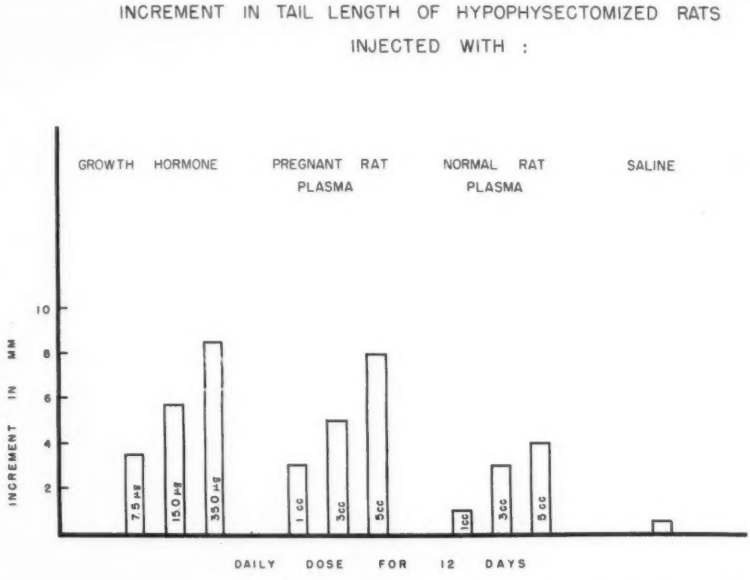

FIG. 4. Comparison in the tail length increment of hypophysectomized rats injected with purified bovine pituitary growth hormone, pregnant rat plasma, normal rat plasma or saline.

purified growth hormone. The tail length increase of hypophysectomized rats parabiotically united with normal rats was comparable to that resulting from injection of 70 μg. of purified growth hormone. From these values, the weight of the animals, the rate of exchange of plasma (13), and the plasma volume (14), it can be calculated that the concentration of growth promoting substance(s) in plasma was 3.5 μg. per cc. This value obtained from parabionts receiving plasma continuously and intravenously does not differ markedly from the value obtained here from injection of plasma, of 1.0 to 2.5 μg./cc. The donors in the experiments reported here were 90–120 days of age, whereas the rats united parabiotically were younger and might be expected to have a higher circulating growth hormone content in plasma.

It has been shown that the growth promoting substances in plasma, at least in the male rat, vary with age, the highest content occurring between 20–50 days of age (15).

Thyroid hormone and growth hormone are the substances to which attention would first be directed in accounting for the growth promoting potency of plasma. In order to examine the role of the thyroid, the growth promoting potency of the plasma of thyroidectomized-pregnant rats has been examined. Thyroidectomy in the first day of pregnancy has not been found to alter the growth promoting activity of pregnant rat plasma (16). When thyroidectomy was performed on non-pregnant female rats the growth promoting activity was decreased, not being detectable at the highest level injected (16). Details of these experiments will be given elsewhere, however, these and other experiments indicate that the maternal and fetal pituitaries and placenta are all involved in the increase in growth promoting potency of pregnancy plasma.

Since increased activity is present early in pregnancy the fetus is probably not involved at this time. The fetal pituitary however, contains growth hormone activity (17) in the later part of pregnancy, so that its participation towards the end of gestation cannot be excluded. The placenta must be considered as it has been shown to produce or contain trophic hormones, similar to pituitary hormones (18). Furthermore, preliminary experiments have shown that extracts of rat placenta increase the tibial epiphyseal width and cause significant increases in the body weight and tail length. It has not yet been clarified when the placenta starts producing this growth promoting activity. The role of the maternal pituitary is not yet understood. The content of growth promoting activity in the rat pituitary does not appear to change during pregnancy (19). This result, however, must be interpreted in the light of possible changes in the production-release mechanism of the pregnant rat pituitary.

SUMMARY

The normal rat plasma contains growth promoting substance(s) as judged by the increase in the tibial width, body weight and tail length of the injected hypophysectomized rats. This activity in normal plasma is equivalent to 1–2.5 μg. of purified bovine growth hormone per cc. of plasma.

The growth promoting activity is increased during pregnancy, to an equivalent of 3.5–7.5 μg. of purified bovine growth hormone per cc. of plasma, a 3-fold increase.

No evidence of TSH in the plasma of normal or pregnant rats was found as judged by the histology, I^{131} uptake and weight of the thyroid of injected hypophysectomized rats.

The role of the maternal and fetal pituitary, the placenta and thyroid in causing this increased activity is discussed.

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LIVER GLYCOGEN DEPOSITION AFTER INTRAVENOUS AND INTRAGASTRIC ADMINISTRATION OF CORTISOL-4-C^{14} TO RATS[1]

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IN THE attempt to correlate the routes by which cortisol-4-C^{14} [3] was administered with the types of radiometabolites excreted (1), the question arose as to the time required to elicit the biological response to the hormone. Furthermore, it was not known whether the corticoids triggered or participated in the reactions producing glycogen deposition in the liver. Winternitz and Long (2) reported that 2 injections of an adrenal cortical extract caused an increase in glycogen content in the livers of adrenalectomized animals in 2 to 4 hours. Ashmore and co-workers (3) showed that the subcutaneous injection of 17-hydroxycorticosterone appeared to increase the conversion of pyruvate to glucose in liver slices from adrenalectomized-diabetic rats within 2 hours and reached a maximum in 6 hours. Other investigators (1,4) have found that following intravenous or subcutaneous injection of radiocortisol approximately 85% of the C^{14} given was excreted in the bile in the first 4 hours in rats with biliary fistulas. Therefore, it seemed that an experiment using both the radiohormone and measuring its biological response in the same animals might delineate its mechanism of action.

Accordingly, fasting animals were administered radioactive 17-hydroxycorticosterone or a control solution and the times required for a significant and maximum deposition of liver glycogen were determined. The intravenous and intragastric routes were selected because they represent two widely varying pathways for absorption and transport of the hormone to the liver and would presumably give different degrees of response. The amounts of radioactivity present in liver and intestinal tract were measured in order to determine the location of the cortisol-4-C^{14} or its metabolites at the various time-intervals.

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[3] Obtained through the kindness of the Radioactive Steroids Allocations Committee of the Endocrinology Study Section, National Institutes of Health, Bethesda 14, Maryland.

METHODS

Male Sprague-Dawley rats, weighing from 280 to 325 gm., were fasted for 22 hours, then allowed Purina chow *ad lib.* for 2 hours following the procedure of Higgins *et al.* (5). After two days of this treatment, the animals were fasted for 24 hours, weighing from 240 to 300 gm. at this time. They were then given the hormone while under light ether anesthesia. In the intragastric series, each of the 30 experimental animals was given 3.0 mg. cortisol per Kg. by stomach tube. The cortisol solution contained 0.9 mg. cortisol and 0.07 μc. of radiocortisol per ml. of 20% ethanol. At the same time, the 33 control animals were given an equal volume, 3.3 ml. per Kg., of a 20% ethanol solution by gavage. For the intravenous series, each experimental animal received 2.50 ml. per Kg. of a steroid solution containing 1.2 mg. cortisol and 0.075 μc. cortisol-4-C^{14} per ml. of 20% ethanol in saline. The control animals were injected with 2.50 ml. per Kg. of a solution of 20% ethanol in saline. Thus the control and experimental animals received the same proportionate amount of ethanol. All of the animals in each absorption series were injected and utilized in a single day, so that they had approximately the same nutritional status (6). Three control animals were sacrificed by having their neck severed at zero time. Thereafter three control and three experimental animals were decapitated at specific time intervals; namely, 5 and 30 minutes, 1, 2, 3, 4, 6, 8, 10, and 12 hours. A lobe of liver from each rat was removed immediately, sectioned, and duplicate samples of it were weighed on a torsion balance. The tissue was digested in 30% KOH according to the procedure of Good *et al.* (7) and the glycogen content determined using the modified anthrone reagent (8). The values found were expressed as glucose equivalents per gm. of wet liver. After the major vessels of each experimental animal had been severed, a small quantity of blood was allowed to drain into a heparinized beaker and the blood volume was measured. The radioactivity in the blood was removed by an extraction technique previously described (9). The remaining liver and intestinal tract and contents were removed, homogenized separately and extracted by the same procedure. Aliquots of these extracts were oxidized to CO_2, precipitated as $BaCO_3$, and counted as described (1).

RESULTS

The liver glycogen contents of the experimental and control animals following intragastric administration of cortisol are shown in Figure 1A. Each point on the graphs is the mean value for 3 animals; the range of values being within the brackets. The amount of liver glycogen present in the experimental animals, 50 μM, was strikingly different from that of the controls, 4 μM, 4 hours after giving the hormone. This difference persisted for at least 8 hours more and reached a maximum at the 10th hour. Figure 1B shows the quantities of radioactivity present in the liver and in the intestinal tract and contents of the experimental animals at various times. The amount of C^{14} found in the liver after 3 hours was 0.5% of the dose, or less, which is the lower limit of measurement by the techniques employed. Radiocarbon was found in the blood samples in measurable quantities at 1 and 2 hours only.

Figure 2A demonstrates that 3 hours after the intravenous injection of cortisol, the liver glycogen content was 17 μM greater than that found in control animals. The difference in glycogen levels was greatest at 4 hours. The blood levels of cortisol-4-C^{14} or its radiometabolites were highest at 5

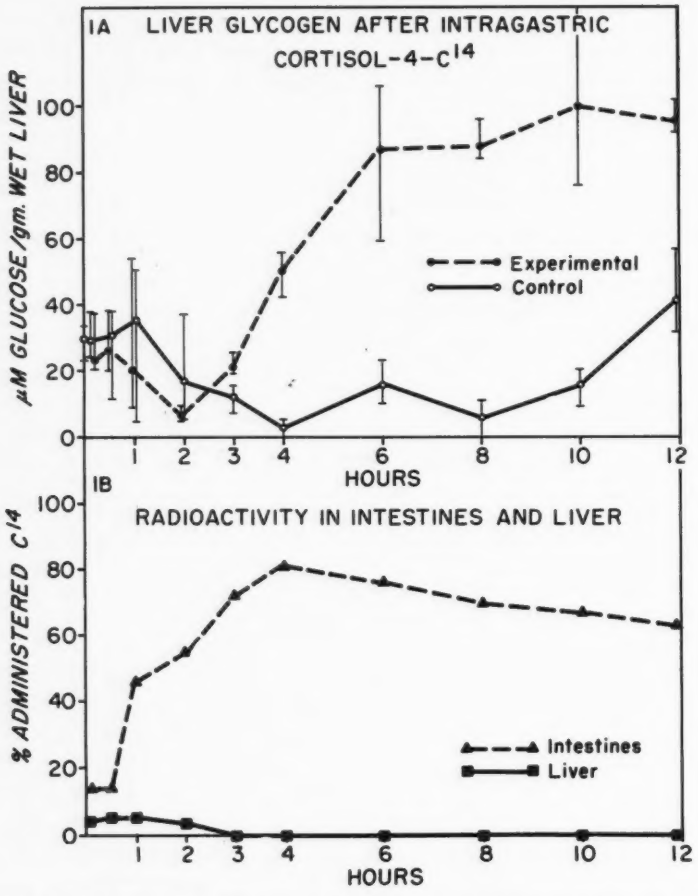

FIG. 1. Response and distribution of C^{14} following intragastric administration of 3 mg. cortisol-4-C^{14}/kg. Each liver glycogen value shown in 1A is the average for 3 animals, the range being within brackets. 1B represents C^{14} found in livers and in intestinal tracts of the corresponding animals.

minutes after injection and disappeared after 2 hours. The concentration of radioactivity in blood samples 1 hour after intravenous administration was from 2 to 3 times greater than that found following gavage. These data were reflected in the concentrations of C^{14} found in the liver: 9% at 1 hour, as contrasted to 5% at the same time for the intragastric series. Only 0.7% of the administered radioactivity was found in the livers 2 hours after parenteral injection and no detectable amounts were found thereafter. Figure 2B illustrates the rapidity with which the liver excretes the injected C^{14} into the intestinal tract, where an average of 24% of the dose was found after 30 minutes. The sudden drop in radioactivity in the

intestinal tract at 4 hours may possibly have been due to enterohepatic circulation of cortisol (1), but no detectable amounts of C^{14} were found in the blood or livers of these animals at this time.

DISCUSSION

Although the deposition of liver glycogen after cortisol injection was apparently initiated earlier than when administered intragastrically, the amount deposited was greater by gavage. Three hours after intravenous injection, the experimental animals had significantly more liver glycogen than the controls, whereas it took 4 hours after gavage. The differences in

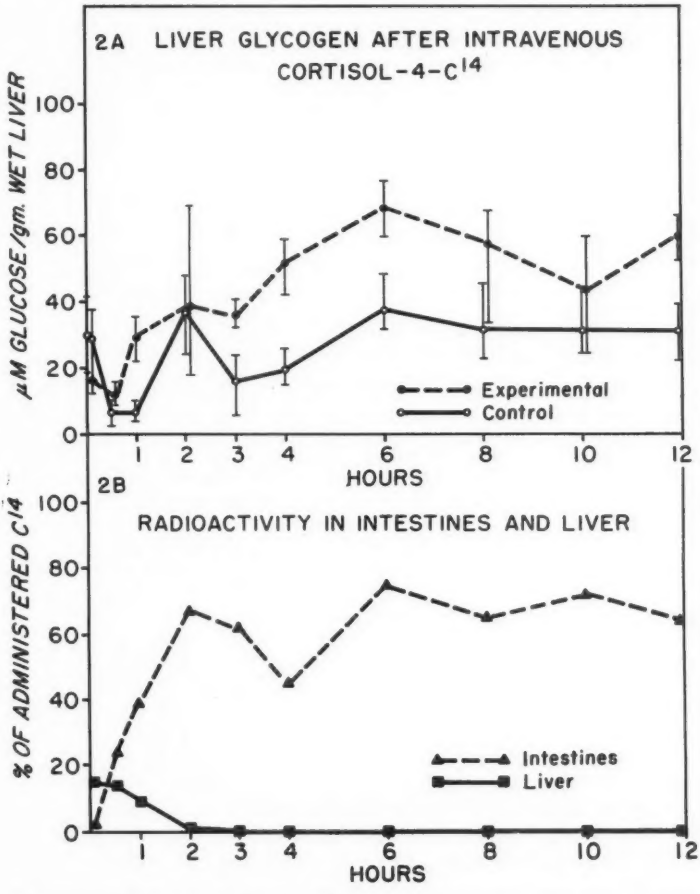

FIG. 2. Response and distribution of C^{14} following intravenous administration of 3 mg. cortisol-4-C^{14}/kg. Each liver glycogen value shown in 2A is the average for 3 animals, the range being within brackets. 2B represents C^{14} found in livers and in intestinal tracts of the corresponding animals.

concentration of radiocarbon in the liver, 5% at 30 minutes following gavage, as contrasted to 14% after intravenous injection may explain the more rapid glycogen deposition. More cortisol or its radiometabolites elicited a faster response. The time response curves to cortisol are in general agreement with the increased incorporation of pyruvate into glucose found at 2 and 6 hours in the livers of adrenalectomized-diabetic rats (3).

The greater amount of glycogen deposited following intragastric rather than after intravenous injection may have been due to many causes. Less of the injected cortisol may have arrived at the liver because of the rapid reduction and inactivation due to enzymatic activity of the blood (10–12). The intravenous injection may have permitted penetration of the hormone into the extracellular spaces, where it could have been metabolized (13) prior to transfer to the liver. In addition, absorption of the cortisol from the intestinal tract via the portal system brings it directly to its site of measurable physiological activity. Porter and Silber (14) have shown that apparent differences in liver glycogen deposition can easily result from differences in absorption of the corticoids.

The data presented in Figures 1 and 2 show that after cortisol or its radiometabolites have been excreted by the liver, approximately an hour elapsed before significant amounts of glycogen were found. Two or more hours were required before a maximum response was obtained. This is in accordance with the concept that cortisol triggers a series of reactions resulting in glycogen formation and deposition in the liver. While as little as 0.5% of the administered steroid could conceivably have been present and participated in the reaction sequence, this is unlikely because the major fraction of the radiosteroid had been transformed into conjugated forms (1) and excreted into the intestine. The best available explanations for this triggering mechanism are that cortisol increases one or a number of enzymes responsible for the conversion of protein or fat into glucose, or that both of these metabolic conversions take place at increased rates.

SUMMARY

Liver glycogen deposition in fasting rats was measured following intravenous and intragastric administration of 3.0 mg. cortisol-4-C^{14} per Kg. Significant increases in glycogen content of the hormone-treated animals over controls were found 3 hours after injection and 4 hours after gavage. Following intragastric administration of radiocortisol, a greater and more prolonged response was noted than that found after intravenous injection. Cortisol-4-C^{14} or its radiometabolites disappeared from the liver approximately one hour before a significant increase in glycogen content took place and 2 or more hours before the maximum physiological response occurred. These experiments suggest that cortisol exerts a triggering action on the reaction sequence producing the deposition of glycogen.

Acknowledgment

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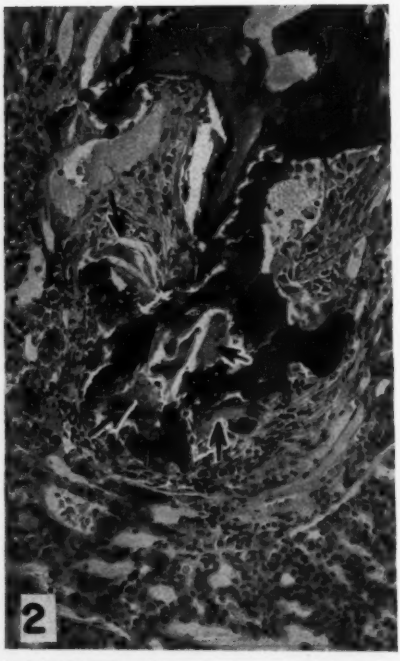

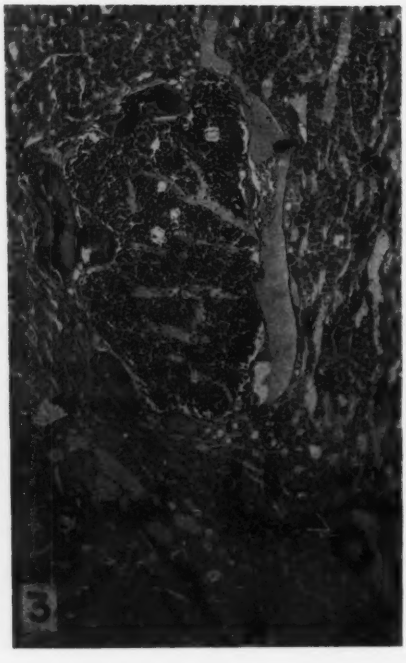

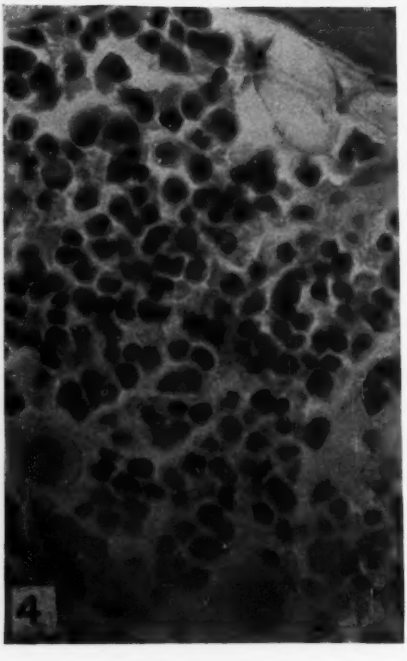

NOTES AND COMMENTS

BONE FORMATION IN THE ADRENAL GLAND OF A RHESUS MONKEY

Ectopic bone has been found in many parts of the body. Kearns (1), in a review on this subject, has noted that there are only a few structures in which this type of bone growth has not been found. It is more prevalent, however, in the cardiovascular system than in other regions and frequently accompanies sclerosis, degenerative changes and other abnormal conditions (1). The majority of these ectopic formations seem to be bony tissue alone. The presence of bony tissue and marrow is less frequent. The adrenal gland is one of the organs in which bone formation is not common; therefore, the description of a small bone found in this organ may be of interest, particularly since it contained marrow which was in a state of active hemopoiesis.

We have studied the adrenal glands (and other organs) of several hundred rhesus monkeys, most of which have been subjected to various experimental procedures.[1] Areas of calcification occur frequently in the cortico-medullary region; these are variable in size and number and may range from one to many. Such zones of calcification have appeared in both normal and experimental monkeys, and are apparently independent of any particular treatment. In our experience, none of the deposits of calcified material has given any indication of bone formation.

In one adrenal of a monkey which had been treated with isoniazid, there appeared a small well-defined cancellous bone with marrow. There was no reason to assume that the treatment was responsible for its formation. This bone was surrounded completely by cortical tissue except in three sections where the most central tip of bone just touched the medulla. However, when seen at a very low magnification, it appears to be located in a region that should be medulla (Fig. 1). If this is so, the bone must have completely replaced this portion of the medulla.

The bone formed a discrete mass. Apparently it was somewhat egg-shaped, for at first and towards the end of the block the sections were elongated or somewhat oval in shape while the intermediate ones were rounded and wider. In its greatest dimensions (Fig. 1) it measured 1.30×0.52 mm.; its maximum thickness was estimated at about 1.5 mm. In sectioning the adrenals for routine study, part of each block was cut, a few sections mounted and the intermittent ones discarded. In this mounted group were twelve sections containing the bone, showing a good sequence of structure from its periphery to its interior. We have estimated that these represented about half of its entire thickness. The remainder of the block of adrenal was then sectioned serially at 6 μ. From this were obtained 128 sections with bone, the last ones being horizontal cuts through the periphery.

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[1] Cooperative studies between The Christ Hospital Institute of Medical Research and The Department of Anatomy, University of Cincinnati College of Medicine.

All sections paraffin, 6 μ thick. Fig. 1 stained with Maximow's hematoxylin-azure-II eosin, Figs. 2–4 with Lillie's hematoxylin and eosin.

FIG. 1. Adrenal gland with the ectopic bone in its greatest dimensions. ×19.

FIG. 2. Horizontal section through the outermost part of the bone, showing periosteum, calcified spicules (possibly), bone with lamellae and osteocytes, and several large osteoclasts (arrows). ×160.

FIG. 3. The bone near its midportion, showing bony spicules around its periphery, typical red marrow, small sinuses and one dilated sinus of the adrenal cortex forming the outermost boundary. Arrows point to the margin of the medulla. ×80.

FIG. 4. Typical red marrow with characteristic hemopoietic elements and megakaryocytes. ×650.

These latter bone sections, and also the ones through the beginning of the block, were covered with a distinct periosteum which contained several rows of fibroblasts. The thickness of the periosteum was probably exaggerated because of the horizontal cut in this region. Here the bony tissue consisted of many spicules close together, with little if any marrow between them. It appeared as though some of these spicules were just calcified material, and not lamellae of bone with osteocytes. This was uncertain, however, since the tissue had not been decalcified and portions of it sectioned poorly. Prevalent in this peripheral region were huge osteoclasts, as many as eight in a section, lying principally between bone and periosteum but also in among the spicules (Fig. 2). Osteoblastic activity was slight or absent.

In subsequent sections through the interior of the bone, a distinct periosteum was usually lacking, the bony tissue or marrow being separated from the adrenal tissue by sinusoids or by fine reticular fibers only. Several small bony spicules were arranged intermittently around the periphery (as in Fig. 3) and others were distributed at random throughout the marrow cavity. The intervening areas were filled completely with dense myeloid tissue consisting of reticular tissue, occasional fat cells, sinusoids, various developmental stages of erythrocytes and granulocytes, some plasma cells, and many megakaryocytes, as in typical femoral marrow (Figs. 3 and 4). Osteoclasts were now generally absent. Slight intermittent osteoblastic activity was seen.

The bone as a whole was surrounded in large part by sinusoids which were continuations of the cortical sinusoids of the adrenal, sometimes considerably dilated (as in Fig. 3). These cortical sinusoids made connections with the sinusoids of the marrow, so that the circulation of the bone was interposed in the cortical circulation of the adrenal.

Aside from the presence of bone, the adrenal was normal in structure. There was no obvious basis for the occurrence of this bone, even as there appears to be no apparent reason for numerous areas of calcification that occur in general in the cortico-medullary region in rhesus monkeys. A discussion of various possibilities for ectopic bone formation is presented by Kearns (1). One can scarcely speculate as to why marrow should also be formed.

A similar instance of bone formation in the adrenal gland of a rhesus monkey was that reported by Kruse (2). This was located in the center of the medulla, was well circumscribed by a periosteum, and appeared to be a typical cancellous bone with marrow, very similar to ours in structure. In other respects the adrenal gland was likewise normal.

The presence of ectopic bony tissue in the adrenal is sufficiently unusual to be of interest. In addition, the striking feature of both Kruse's case and ours was the organization of the bony tissue into a typical cancellous bone with active marrow.

SUMMARY

A small cancellous bone, with marrow in active hemopoiesis, was found in the cortex of an otherwise normal adrenal gland of a rhesus monkey. The unusual feature was the high degree of organization of this bone, differing from the more common random formation of ectopic bone.

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THE ENDOCRINE SOCIETY

1958 ANNUAL MEETING

The Fortieth Annual Meeting of The Endocrine Society will be held in the St. Francis Hotel, San Francisco, California, Thursday, Friday and Saturday, June 19, 20, 21, 1958.

The Committee on Local Arrangements is Dr. Roberto Escamilla, Chairman, Dr. Donald E. Bernstein, Dr. Peter H. Forsham, Dr. Minnie B. Goldberg, Dr. Gilbert S. Gordan, Dr. Francis S. Greenspan, Dr. C. H. Li as members of the committee.

All Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily in the Colonial Ballroom, and in addition there will be simultaneous afternoon sessions in the Italian Room. The annual dinner is scheduled for Friday, June 20th at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All reservations for rooms must be made through the AMA Housing Bureau, c/o Convention and Tourist Bureau, Civic Auditorium, San Francisco, California, after January 1, 1958. No reservations will be accepted before that date. If possible, official application forms as published in the Journal of the American Medical Association should be used. Arrival and departure dates must be stated. *This notice concerning procedure for making hotel reservations supersedes that published in the November 1957 issue of this journal.*

Final program, membership card and advance registration forms will be sent on May 15, 1958 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Rulon W. Rawson, 444 E. 68th St., New York 21, New York, not later than February 1, 1958. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.

2. The title heading must be arranged as follows:

Line 1. Title, not to exceed fifteen words;
Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.
Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

4. Abstracts should be letter perfect, since there will be no opportunity for proofreading by the authors.

THE FOURTH INTERNATIONAL GOITRE CONFERENCE LONDON 1960

The American Goiter Association and the London Thyroid Club announce that the Fourth International Goitre Conference will take place in London from 5th to 9th July, 1960.

Scientific sessions will be held under the presidency of Sir Charles Harington at the Royal College of Surgeons, Lincoln's Inn Fields, W.C.2, on Wednesday, Thursday and Friday, 6th, 7th and 8th July.

Those who desire to submit papers for consideration by the Programme Committee should send abstracts not later than 31st December, 1959, to: Dr. Selwyn Taylor, 3 Roedean Crescent, London, S.W.15, or Dr. John C. McClintock, 149½ Washington Avenue, Albany 10, N. Y., U. S. A.

Hotel reservations may be secured from Messrs. Thomas Cook & Son Limited, and it is anticipated that special travel facilities will be offered by B.O.A.C.

A very few honoraria will be available for travel expenses. For further particulars please write to Dr. Selwyn Taylor or to Dr. J. C. McClintock.

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MODERN THERAPY IN NEUROLOGY. Francis M. Forster. St. Louis, Missouri: The C. V. Mosby Co. 792 pages. $12.00

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DIGESTIVE SYSTEM. Liver, Biliary Tract and Pancreas. No. 2 of Volume Three. The Ciba Collection of Medical Illustrations. Prepared by Frank H. Netter and edited by Ernst Oppenheimer. Publications department, Ciba Pharmaceutical Products, Inc., Summit, New Jersey. 165 pages. $10.50

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RHYTHMES ET CYCLES BIOLOGIQUES. Alain Reinberg and Jean Ghata. Paris: Presses Universitaires de France. 127 pages.

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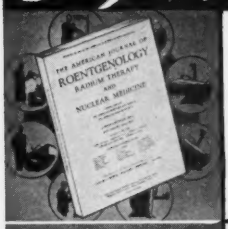

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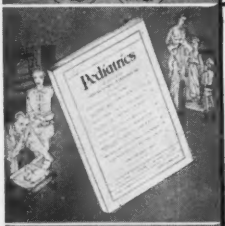

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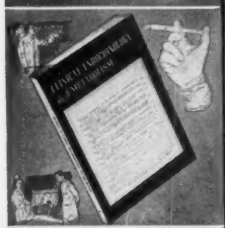

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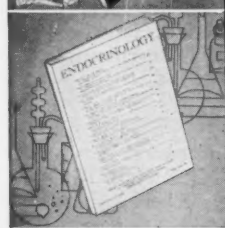

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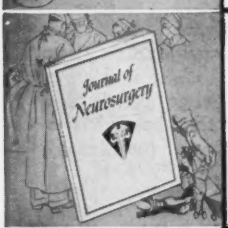

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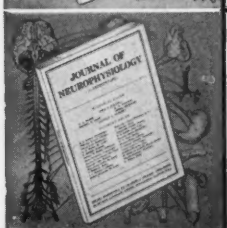

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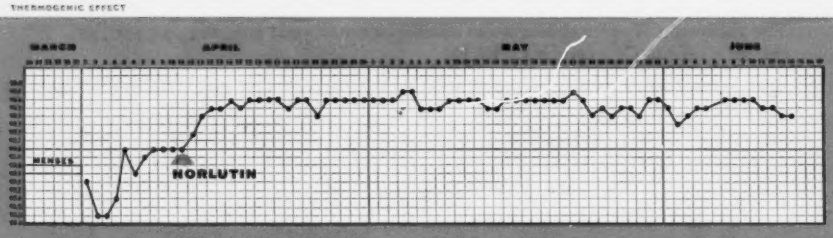

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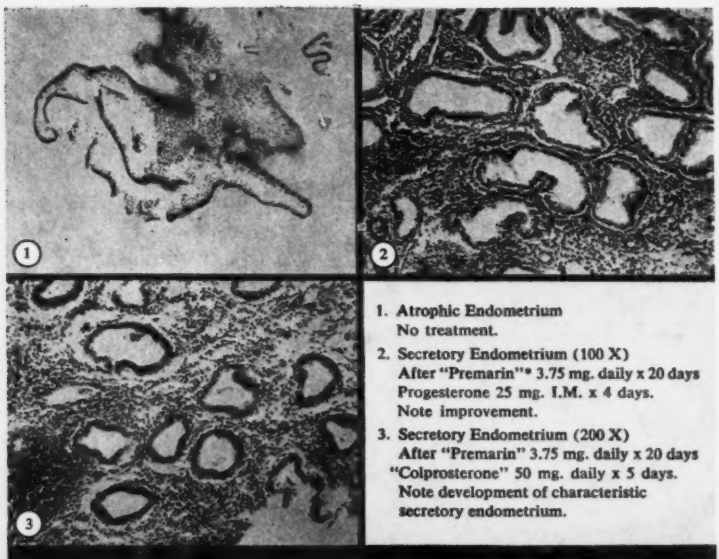

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